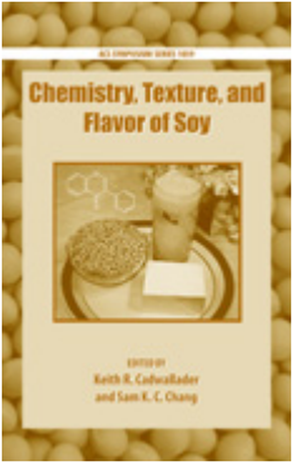


ACS SYMPOSIUM SERIES 1059

Chemistry, Texture, and Flavor of Soy

EDITED BY
Keith R. Cadwallader
and Sam K.-C. Chang

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.fw001

Chemistry, Texture, and Flavor of Soy

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.fw001

ACS SYMPOSIUM SERIES **1059**

Chemistry, Texture, and Flavor of Soy

Keith R. Cadwallader, Editor
Department of Food Science and Human Nutrition,
University of Illinois

Sam K. C. Chang, Editor
Department of Cereal and Food Sciences,
North Dakota State University

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.fw001

Sponsored by the
ACS Division of Agricultural and Food Chemistry

American Chemical Society, Washington, DC

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.fw001

Library of Congress Cataloging-in-Publication Data

Chemistry, texture, and flavor of soy / [edited by] Keith R. Cadwallader, Sam K. C. Chang ; sponsored by the ACS Division of Agricultural and Food Chemistry.
p. cm. -- (ACS symposium series ; 1059)
Includes bibliographical references and index.
ISBN 978-0-8412-2561-9
1. Soyfoods--Congresses. 2. Plant proteins--Congresses. 3. Soy bean--Congresses. 4. Food--Analysis--Congresses. I. Cadwallader, Keith R., 1963- II. Chang, Sam K. C. (Sam Kow-Ching) III. American Chemical Society. Division of Agricultural and Food Chemistry.
TX558.S7C44 2010
664′.805655--dc22
2010048122

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48n1984.

Copyright © 2010 American Chemical Society

Distributed by Oxford University Press

All Rights Reserved. Reprographic copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Act is allowed for internal use only, provided that a per-chapter fee of $40.25 plus $0.75 per page is paid to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA. Republication or reproduction for sale of pages in this book is permitted only under license from ACS. Direct these and other permission requests to ACS Copyright Office, Publications Division, 1155 16th Street, N.W., Washington, DC 20036.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.fw001

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.pr001

Preface

For centuries soy has served as an important and inexpensive source of high quality protein throughout the world, especially in Asia. Soybeans are now cultivated in large scale in North and South America, where they are mainly used for production of edible oil and animal feed. Part of the defatted soy meal by-products are refined into functional ingredients for inclusion into various processed food products, including meat, dairy, wheat, beverage and snack food products. Therefore, the consumption of soy has increased tremendously in the Western countries in the last thirty-forty years. More recent discoveries of a number of health benefits of soy have spurred a new wave of demand for soy foods, not only in Western countries, but also in those countries which traditionally consumed soy. Growth in soybean production and utilization is also occurring in developing countries to help meet nutritional needs of those populations.

Despite the tremendous growth in the global consumption of soy, there are still many technological challenges that must be met to improve the food quality, nutritional and health promoting attributes of soy-containing foods. In particular, numerous flavor and textural challenges impact the quality and consumer acceptability of soy foods. These flavor and texture properties as well as the nutritional and health-promoting attributes are mainly determined by the chemistry and functionality of the chemical components of soy. We hope that this book will serve as a platform for future scientific and technological studies leading to improvements in the quality and acceptability of soy foods.

This book is a culmination of a symposium titled “Chemistry, Texture and Flavor of Soy”, which was sponsored by the Agricultural and Food Chemistry Division of the American Chemical Society and held at the 236th ACS National Meeting in Philadelphia, PA, August 17-21, 2008. Leading national and international academic, government and industrial researchers from several countries have covered the following topics as they relate to the quality of soy foods and ingredients:

- Chemistry of soy and soy components, including isolation and characterization of bioactives and functional ingredients/compounds.
- Texture aspects of soy and soy ingredients, including processing, characterization and measurement by sensory and/or instrumental means.
- Flavor chemistry and analysis (sensory and instrumental) of soy and soy products/ingredients/components.

We are grateful to the authors for their contributions and express our appreciation to the many reviewers for their valuable insights and critiques of the chapters. We acknowledge with great appreciation the financial support from the ACS Division of Agricultural and Food Chemistry, the Illinois Center for Soy Foods (through generous funding from the Illinois Soybean Association) and the North Dakota Soybean Council.

Keith R. Cadwallader
Professor

University of Illinois
Department of Food Science and Human Nutrition
1302 W. Pennsylvania Avenue, Urbana, IL 61801
cadwlldr@uiuc.edu (e-mail)

Sam K. C. Chang
Professor

North Dakota State University
Department of Cereal and Food Sciences
IACC 322, Dept. 7640
Fargo, ND 58108-6050
Kow.chang@ndsu.edu (e-mail)

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.pr001

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Chapter 1

Carbon-Centered Radicals in Soy Protein Products

William L. Boatright*,[1] and M. Shah Jahan[2]

[1]Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40546
[2]Department of Physics, University of Memphis, Memphis, TN 38152
***Wlboat1@pop.uky.edu**

The free-radical content of typical powdered soy protein products ranged from 2.96×10^{14} to 4.10×10^{15} spins per gram. These levels are about 14- to 100-times greater than other food protein sources. The majority of these free radicals appear to be formed after the soy protein product is manufactured, and during storage of the ‘dry’ powder exposed to oxygen. The radicals react to form non-radical species once the soy protein is hydrated with water, except in solutions of erythorbate or cysteine that result in elevated levels of radicals. If the water-hydrated proteins are subsequently dried and again stored exposed to oxygen, the levels of radicals will gradually increase back to levels that existed prior to hydration. Based on the peak shape, g-value and power saturation characteristics obtained with electron paramagnetic resonance spectroscopy, the free radicals in soy protein are predominately comprised of carbon-centered radicals.

Free-radicals in food products have been studied extensively because of their contribution to deteriorative-type reactions; both in foods during storage and in humans. Generally, free radicals in biological materials are very short-lived. Oxygen radicals have typical half-lives ranging from 10^{-9} seconds for a hydroxyl radical (OH•) to 7 seconds for a lipid peroxy radical (LOO•) (*1*). In solution, the stability of alkyl radicals generally follow the order of tert-alkyl > sec-alkyl > n-alkyl > methyl, with the methyl radical having a half-life of 0.2×10^{-3}

seconds (*2*). Hydroxyl radicals have been shown to react with proteins to produce carbon-centered radicals. These carbon-centered radicals are short-lived in solution and react with a variety of compounds to produce non-radicals species including protein–protein crosslinks (*3*), DNA adducts (*4*, *5*), DNA strand scission (*6*, *7*) and protein strand scission (*8*, *9*).

The direct detection and quantification of carbon-centered radicals in solution has proven difficult because their half-lives are in microseconds, and because they can react with oxygen at a diffusion-controlled rate to form secondary radicals that are oxygen-centered. Spin-trap adducts can aid in the analyses of these short lived radicals. The spin-trap adducts of carbon-radicals had shorter half-lives (from 3.1 to 8.4 min) than corresponding adducts formed from hydroxyl- or sulfur-centered radicals (*10*).

Certain purified proteins in the 'dry' state, exposed to ionizing irradiation, were found to act as free-radical traps (*11*, *12*). Using electron paramagnetic resonance spectroscopy (EPR), Uchiyama and Uchiyama (*13*) found that pyrolysis of protein-rich foods, including 'dry' soy protein and individual amino acids, resulted in the formation of free-radicals (g = 2.0030-2.0049). Lee and others (*14*) and Huang and others (*15*) identified a central singlet signal in soy protein (g = 2.00412.0054) as being from carbon-radicals, based on the observations of Pshezhetskii and others, and Henriksen and others (*16*, *17*). Uchiyama and Uchiyama (*13*), Lee and others (*14*) and Huang and others (*15*) all examined soy protein samples that had been exposed to some type of treatment (pyrolysis, γ-irradiation or lipoxygenase activity; respectively). None of these studies reported the type or level of free-radicals in soy proteins that had only been exposed to processing and storage conditions typically encountered with proteins use for human foods.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Free Radicals in Typical Commercial Soy Protein Products

Boatright and others (*18*) presented an EPR spectrum of a typical commercial ISP sample at 1 mW power (Figure 1A). The large symmetrical peak (g = 2.005) was typical for radicals localized on the carbons of amino acids (*16*, *17*). Also, a plot of signal amplitude vs. square root of the power revealed that the microwave power was at non-saturating levels for the primary radical signal (g = 2.005) up to slightly above 4 mW. Only the carbon radical produces an EPR spectra with these characteristics of peak shape, g-value and power saturation level.

For comparison, the EPR spectra of three other common food proteins (casein, sodium caseinate and egg albumin) along with a sample of rancid soybean oil (PV=16 ± 0.0) are presented (Figure 1, B-E). The level of free-radicals in the 'dry' (or powdered) protein samples were estimated using a standard curve of powdered K_3CrO_8 in K_3NbO_8 prepared by the method of Cage and others (*19*) and diluted with powdered KCl. The Cr(V) spin concentrations were calculated from a standard curve of the ESR signal of Fremy's salt (dipotassium nitrosodisulfonate) solutions at −196°C after double integration. The free-radical content of the commercial sample was 14-times greater than that of similar radicals trapped in sodium caseinate, 29-times greater than egg albumin, and about 100-times greater than in casein (the casein peak being too small to accurately integrate). There was no detectable signal from the rancid soybean oil.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

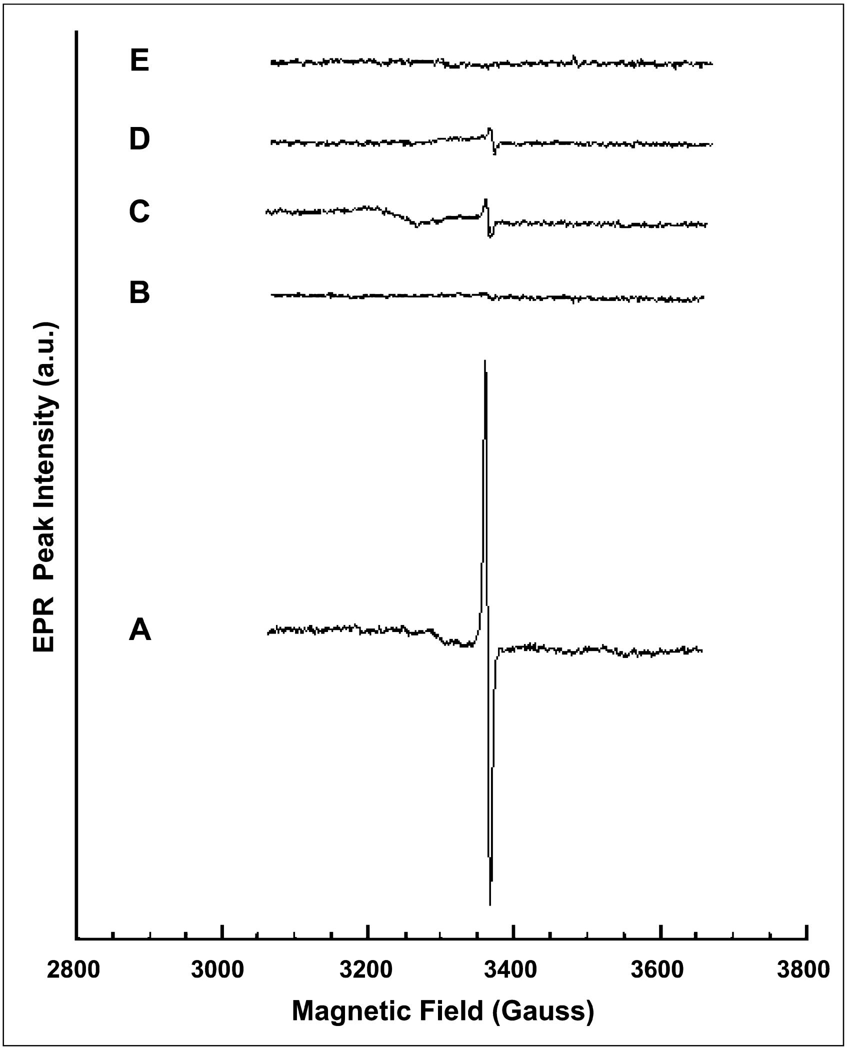


Figure 1. EPR spectra of A) commercial ISP, B) casein, C) sodium caseinate, D) egg albumin, and E) rancid soybean oil. All protein samples are in the solid state and all are at 1mW. Primary free radical at g = 2.005. (Reproduced from Reference (18), ©2008 John Wiley and Sons.)

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Table I. Peak Area after Double Integration of Primary EPR Peak in Commercial Isolated Soy Protein Samples and Commercial Soy Protein Powdered Drink Mixes*

Product	*Peak Area* × 10^5
Commercial ISP A	5.53
Commercial ISP B	3.40
Commercial ISP C	4.74
Commercial ISP D	3.97
Laboratory ISP	4.16
Nature's Life Super Blue	5.67
Nature's Life Super Green	11.41
Universal Nutrition SoyPro Chocolate Flavor	23.05
Solaray Soytein Chocolate Peanut Butter Flavor	12.61
Solaray Soytein Vanilla Flavor	4.96
Solaray Soytein Natural Flavor	11.38
GenSoy Chocolate Flavor	38.57[a]
GenSoy Vanilla Flavor	16.28
GenSoy Natural Flavor	16.23

[a] Estimated spin-concentration (spins per gram of soy protein product) = 4.10 × 10^{15}. * SOURCE: Reproduced from Reference (*18*), ©2008 John Wiley and Sons.

The free-radical concentration in commercial powdered protein drinks (with ISP as the main ingredient), along with several samples of commercial ISP and a laboratory prepared ISP, was also reported by Boatright and others (*18*) (Table I). The commercial and laboratory ISP contained similar levels of radicals (ranging from 2.96 × 10^{14} to 6.42 × 10^{14} spins per gram), while the powdered soy protein drinks ranged from about 1 to 6.4 times greater than the highest level found in commercial ISP samples. The soy protein drink mix with the most intense EPR peak was estimated to contain 4.10 × 10^{15} spins per gram of drink mix (Table I). This was the first time that the level of paramagnetic centers in soy protein products had been reported.

Boatright and others (*20*) demonstrated that retail samples of ISP, soy protein concentrate (SPC) and powdered soy milk all had levels of carbon-centered radicals (Table II) from about one to four times as high as the levels previously reported for ISP samples obtained directly from the processor (Table I). All ISP, SPC and powdered soy milk samples examined contained carbon-centered free radicals from 3.5- to 12-times higher than whey proteins (Table II; Figure 2). The free-radical content of whey protein samples examined were similar to those reported for egg albumin and sodium caseinate (*18*). Roasted soy nuts contained about 5.70 × 10^{15} carbon-centered radicals per gram. The peak-to-peak line width of the first derivative curve of the carbon-centered radicals from soy nuts was about 10 gauss, while ISP samples with a similar peak height had a peak width of about 8 gauss. Kosuge and others (*21*) and Uyeta and others (*22*) reported on the formation of mutagenic substances formed from the pyrolysis of proteins. Uchiyama and Uchiyama (*13*) demonstrated that heating soy protein above 75°C for one hour elevated the level of free radicals at g = 2.0037–2.0045. The broader EPR peak in soy nuts resulting from pyrolysis indicates that the higher temperature process is more random than the stable-radical forming process that occurs over time at room temperature. Retail snack bars containing ISP, SPC, and/or roasted soy nuts with a total protein content of either 13, 21 or 29 percent contained 5.32×10^{14}, 6.67×10^{14} and 5.74×10^{14} spins per gram of snack bar, respectively (Table II). These are levels similar to that reported in some ISP samples (at about 85 percent protein).

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

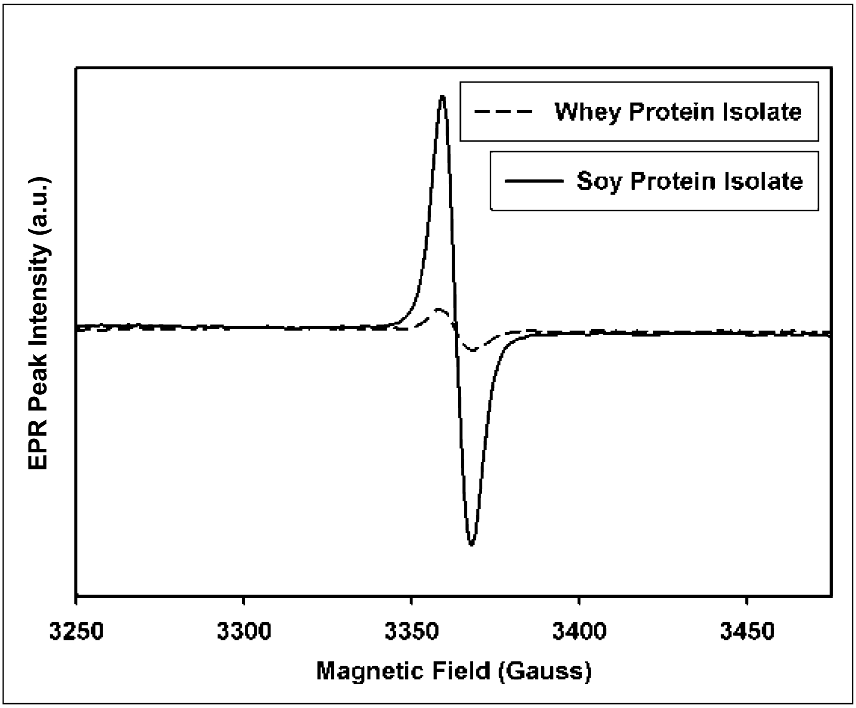


Figure 2. EPR spectra from a whey protein isolate (GNC brand) and a soy protein isolate (Now brand). Peak areas are provide in Table II. Both are in the solid-state at 1mW power. (Reproduced from Reference (20), ©2009 American Chemical Society.)

Table II. Peak Areas after Double Integration of Primary EPR Signal in Laboratory Isolated Soy Protein Samples and Various Protein Products*

Product	*Peak Area × 10⁵*
Protein Products from Local Markets:	
Now ISP	15.28
Bulk Foods ISP	14.44
Life Extension Soy Protein Concentrate	5.24[a]
The Sausage Makers Soy Protein Concentrate	18.34[b]
Now Powdered Soy milk	16.65
Roasted Soy Nuts	53.86[c]
ON ‘100% Whey Protein’	1.51
GNC ‘100% Whey Protein’	1.58
29% Protein Bar (Kraft South Beach Living - Peanut Butter)	4.87
21% Protein Bar (Luna S’mores)	5.90
13% Protein Bar (SoyJoy Apple)	4.58
Laboratory ISP, all analyzed within 1 day of preparation:	
Lab ISP Extracted at 22°C, pH 9	0.48
Lab ISP Extracted at 50°C, pH 8	1.54
Lab ISP Extracted at 50°C, pH 9	0.99
Lab ISP Extracted at 50°C, pH 9 - held 30 min before freezing	1.47
Lab ISP Extracted at 50°C, pH 8.5	0.78

[a] Estimated Spin Concentration (spins per gram of soy protein) = 6.12×10^{14}. [b] Estimated Spin Concentration (spins per gram of soy protein) = 1.98×10^{15}. [c] Estimated Spin Concentration (spins per gram of soy protein) = 5.70×10^{15}. * SOURCE: Reproduced from Reference (*20*), ©2009 American Chemical Society.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Free Radicals Formed during Storage of Powdered Soy Protein

The level of free radicals in proteins was reported to be elevated by exposing the protein to a mixture of lipoxygenase and linoleic acid (*15*). Because of this, ISP process variations that might affect the ability of naturally-occurring lipoxygenase to contribute to the carbon-centered radicals of ISP were investigated. Laboratory ISP samples were processed at either 22°C or 50°C, using an extraction pH at either 8 or 9, and varying the time the protein slurry was held before freezing at either 0 or 30 min. Regardless of the treatment, all laboratory ISP samples analyzed within 48 hours of being dried had carbon-centered free radicals contents at about one-tenth to one-thirtieth the level of previously analyzed laboratory ISP and commercial ISP samples (Table II) (*1*). Huang and others (*15*) employed high levels of lipoxygenase/substrate at the optimal pH for enzymatic activity (pH 9), and the reaction mixture was exposed to a flash freezing to help trap the radicals.

Also, because there was no quantitative data provided, no comparisons of the levels of radicals trapped in commercial ISP samples can be made. While their findings do indicate that some protein oxidation occurs as a result of oxidizing lipids during ISP processing, there appears to be another mechanism responsible for the elevated levels of carbon-centered free radicals in powdered commercial ISP products.

After laboratory ISP samples had been stored in the dark at 22°C in sealed 500 mL jars (with a large air headspace), it was observed that the level of free-radicals increased by as much as 35-fold during the first 9 weeks of storage (Figure 3). Storing the ISP under a nitrogen headspace was found to inhibit the increase in radicals over time compared to ISP stored with an air headspace (Figure 4). The jar containing the ISP stored under nitrogen was opened periodically to remove enough samples for EPR analysis which allowed some oxygen to contact the sample. The effect of oxygen on the free-radicals in ISP was further investigated by preparing a single batch of ISP and dividing it into three portions, with one portion stored exposed to air at 23°C, one portion stored under nitrogen at 23°C, and a portion stored under 99.9 percent oxygen at 40°C (Figure 5). A temperature of 40° was chosen to accelerate oxidation processes without significantly altering the protein structure. The level of carbon-centered radicals in the ISP stored under nitrogen remained nearly constant, while the sample exposed to air increased similarly to the control samples exposed to air (Figure 3), and the sample exposed to oxygen at 40°C demonstrated a more rapid increase in radical content. This experiment was repeated with a single batch of ISP divided between 99.99% oxygen, air and nitrogen, but with all portions stored at 23°C (Figure 6). A similar trend was observed indicating the accelerating effect of a higher oxygen content.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

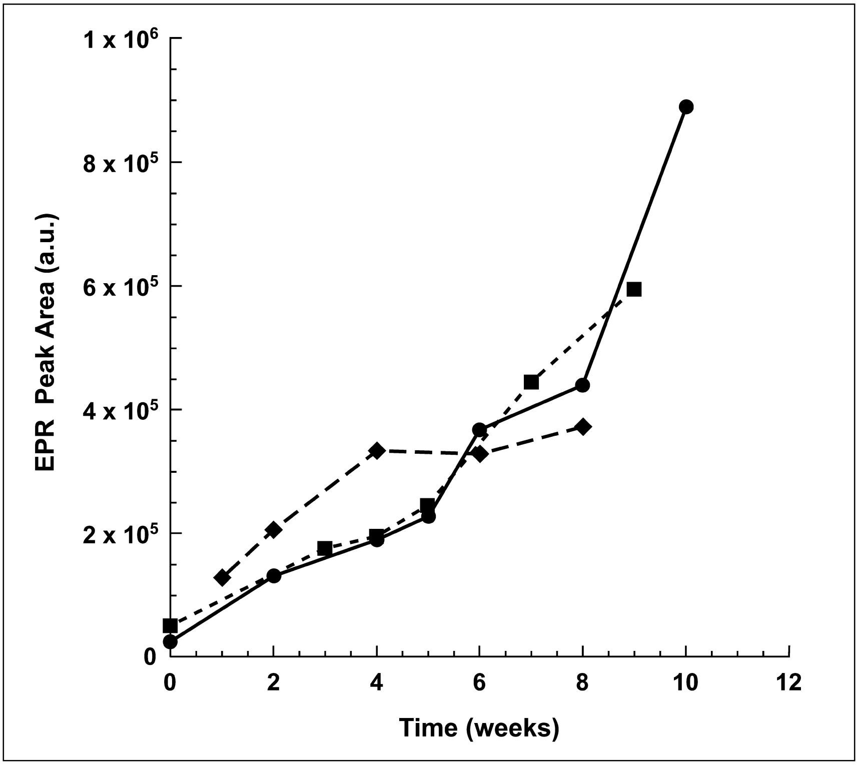


Figure 3. EPR peak area (after double integration) over time for the level of carbon-centered radicals in three different laboratory ISP samples exposed to air at 23°C (♦,▪,•). (Reproduced from Reference (20), ©2009 American Chemical Society.)

These results demonstrate that in the presence of oxygen, the rate of radical formation is accelerated. The reaction mechanism involving oxygen in the ‘dry’ soy protein may proceed similarly to previously published pathways. These may include the formation of superoxide radical anions and subsequent pathways, involving the relatively high content of iron in soy proteins, leading to hydrogen peroxide and hydroxyl radicals. Also the reaction of oxygen with carbon radicals leading to hydroperoxides, alkoxy radicals, carbon-carbon bond cleavage and the subsequent propagation of free-radical reactions, is likely (*23*, *24*). However, most published pathways involving oxygen, that either react with free radicals or that produce free radicals occur in an aqueous environment (*25*). Commercial and laboratory ISP samples typically have a moisture content of 7 to 8 percent (*26*, *27*) and a corresponding water activity of about 0.2. The diffusion of reactions under these conditions would be severely impeded. Because oxygen readily reacts with protein-carbon radicals forming protein-peroxy radicals (*28*), how then can the carbon-centered radical content of soy protein be so stable, and rise to such high levels when stored under oxygen? This question remains to be answered. However, because the levels of carbon-centered radicals are clearly being elevated to high levels, there must be some stearic hindrance or other means of stabilization to protect the carbon-radicals lodged deep within the proteins. Soy proteins appear to be particularly suited to accomplish this.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

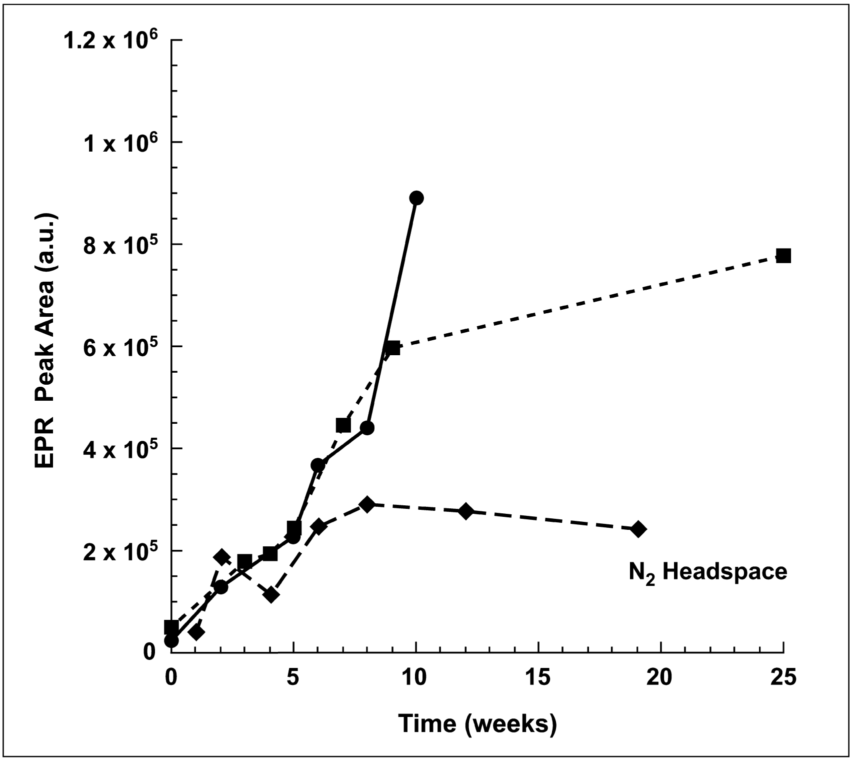


Figure 4. EPR peak area over time for the carbon-centered radicals in two laboratory ISP samples exposed to air at 22°C (•,▪), and another ISP sample stored under nitrogen (♦). (Reproduced from Reference (20), ©2009 American Chemical Society.)

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

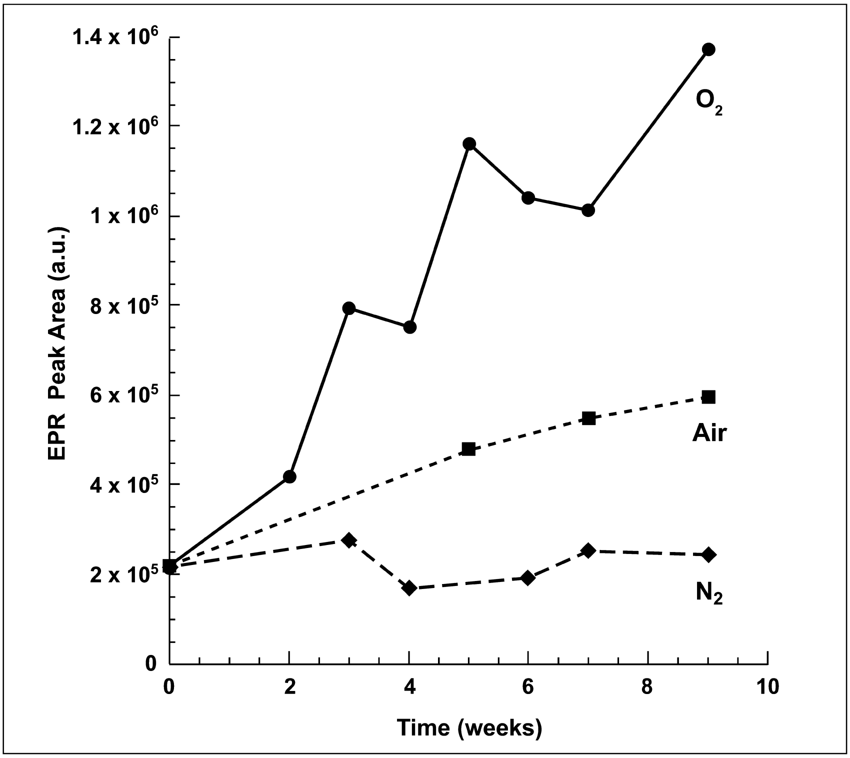


Figure 5. EPR peak area over time for the carbon-centered radicals in a single batch of laboratory ISP with one portion exposed to air at 23°C (▪), another portion stored under nitrogen at 23°C (♦), and a third portion stored under 99.9 percent oxygen at 40°C (•). (Reproduced from Reference (20), ©2009 American Chemical Society.)

Boatright and others (*18*) noted that when ISP samples were hydrated, stirred for 30 min, and then rapidly frozen and dried, the level of free radical in the protein was reduced by about 80 percent (Table III). The effect of hydration, leading to an alteration of the protein conformation and a decrease in trapped radicals, was previously reported for myosin (*29*) and for powdered soybean axes and cotyledons (*30*). The hydration experiment was repeated (at 22°C) to determine if, after the initial loss of trapped radicals from the soy protein, the free radicals would gradually increase again during storage exposed to the air. This was also done at 92°C to determine if thermally denaturing soy protein affected its ability to trap radicals (Figure 7). The ISP sample used initially had a carbon-centered radical peak area (after double integration) of 14.44 × 10^5 before being hydrated. Thus the hydration process resulted in a loss of approximately 91% and 92.5% of the trapped radicals by the 22°C and 92°C treatments, respectively. The level of carbon-centered radicals in these same ISP samples immediately began to increase during subsequent storage exposed to air, at a rate similar to that observed in newly prepared samples. The 92°C treatment appeared to have no significant effect on the ability of radicals to be formed during subsequent storage, compared to the 22°C treatment. Thus, soy proteins in food products, with low water activity (whether or not they were processed in an aqueous environment), can lead to unintentional consumption of high levels of potentially harmful free-radicals.

When a commercial ISP was suspended in 2.3 mM sodium erythrobate (Table III), the free-radical signal (g = 2.005) increased by 17-fold, compared to the rehydrated control which was about 2.5 times higher than the non-hydrated control. Adding 8.3 mM L-cysteine caused a 19-fold increase, while other reducing agents (sulfite (9.7 mM) and dithiothreitol (6.5 mM)) caused little change in the primary radical signal. The cysteine/ISP mixture produced a single EPR peak (g = 2.005) with a peak-to-peak line width of the first derivative curve that was 10.2 Gauss wide. All other peaks observed at 2.005 g were 8 Gauss. Broad peaks are typical of overlapping signals in polycrystalline or powdered samples. All of the reducing agents examined have been shown to catalyze the oxidation of methionine and/or linoleic acid when added to ISP aqueous slurries (*31*, *32*). The difference in their ability to cause the formation of stable carbon-centered radicals within soy proteins may relate to the type of free radical they initially produce. For example, a dithiothreitol free-radicals or sulfite free-radicals may tend to react with components of ISP in solution that do not lead to trapped free-radicals. Erythorbate likely reacts with the high iron and copper content of soy-protein products (*33*), and promotes the formation of hydroxyl radicals, similar to ascorbate (*34*). A common ingredient in the powdered soy protein drinks examined was ascorbic acid, which may further contribute to the elevated free-radical content once these powders are hydrated.

Table III. Effect of Hydrating Soy Proteins, With or Without Reducing Agents, Followed by Rapid Freezing and Drying on the Level of Radicals in the Solid State[*]

Product/Treatment	*Peak Area × 10^{5a}*
Commercial ISP	3.97
ISP hydrated with water	0.54
ISP hydrated with 2.3 mM sodium erythorbate	9.33
ISP hydrated with 8.3 mM L-cysteine	10.25
ISP hydrated with 6.5 mM dithiothreitol	0.47
ISP hydrated with 9.7 mM sodium sulfite	0.64

[a] Peak area after double integration of primary free radical (g = 2.005). [*] SOURCE: Reproduced from Reference (*18*), ©2008 John Wiley and Sons.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

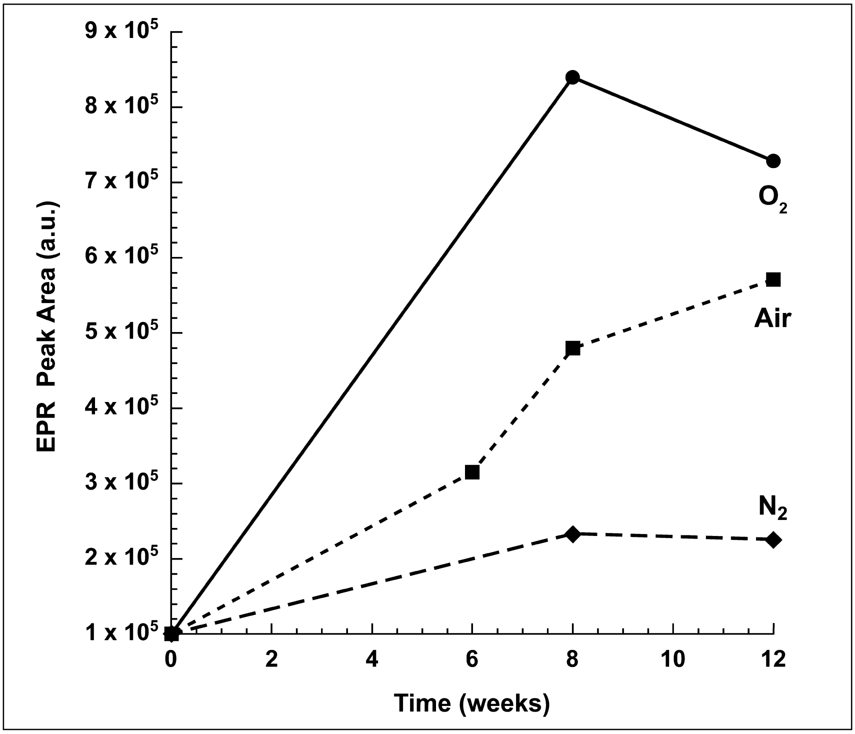


Figure 6. EPR peak area over time for the carbon-centered radicals in a single batch of laboratory ISP with all portions held at 23°C. One portion was stored in air (▪), another portion was stored under nitrogen (♦) and a third portion was stored under 99.9 percent oxygen (•). (Reproduced from Reference (20), ©2009 American Chemical Society.)

Examination of hexane-defatted soybean flour (the raw material used to prepare ISP) revealed another important EPR signal: a strong sextet pattern typical of manganese-II (*35*, *36*) overlapping the primary free-radical peak (Figure 8A). Examination of the hexane-defatted flour at 20 mW power was used to enhance the manganese signal (Figure 8(B-C)). Manganese in the laboratory ISP at 1 mW was not evident (Figure 8(B-C)). At 20 mW the manganese signal in the laboratory ISP sample was clearly evident (Figure 9A), but it was faint from the commercial ISP at 20 mW (Figure 9B). Because the manganese content in ISP is typically about one-half of that found in defatted soy flour (*33*), the approximately 20-fold decrease in the manganese EPR signal in the ISP samples (compared to the defatted flour) may be due to a change in the oxidation state of manganese (*36*), or a change in the association of manganese with other components. Manganese can react with the naturally-occurring sulfite content of ISP, in the presence of oxygen, to produce methanethiol from the one-electron oxidation of methionine (*32*). Finally, there were no sulfur-centered radicals observed in powdered laboratory ISP at power levels ranging from 1 to 160 mW, based on the absence of a signal at g_1= 2.003, g_2= 2.025-2.028 and g_3= 2.062-2.064 (*12*, *37*). Increased manganese signals at the higher microwave powers overlapped the signal location of potential sulfur-centered radicals, making identification of low levels of sulfur-centered radical signal difficult.

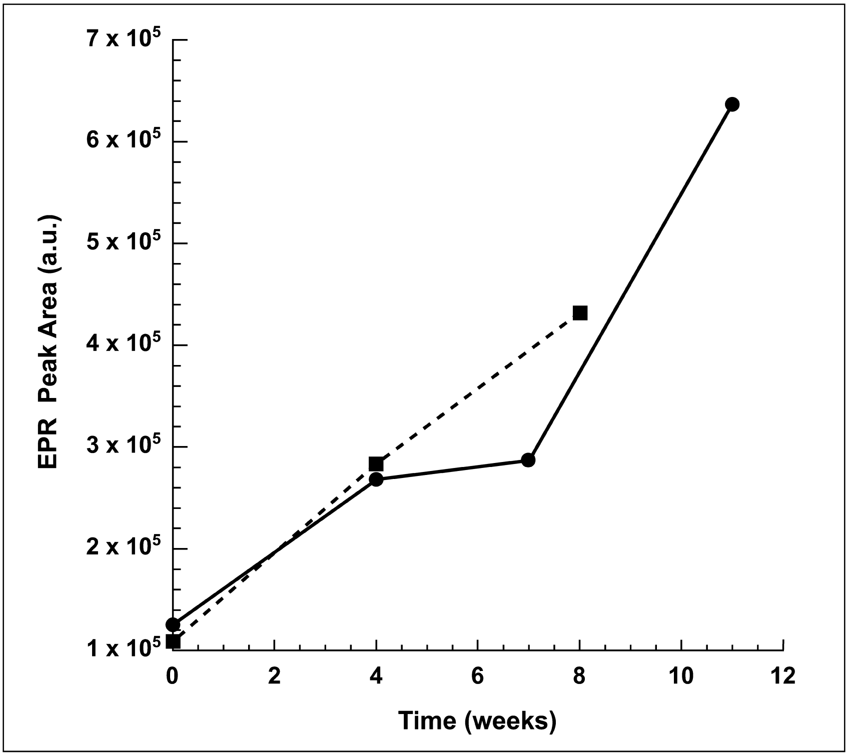


Figure 7. EPR peak area over time for the carbon-centered radicals in ISP after being hydrated at either 22°C (•) or 92°C (▪), stirred for 30 min, and freeze-dried followed by storage in air at 22°C. (Reproduced from Reference (20), ©2009 American Chemical Society.)

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

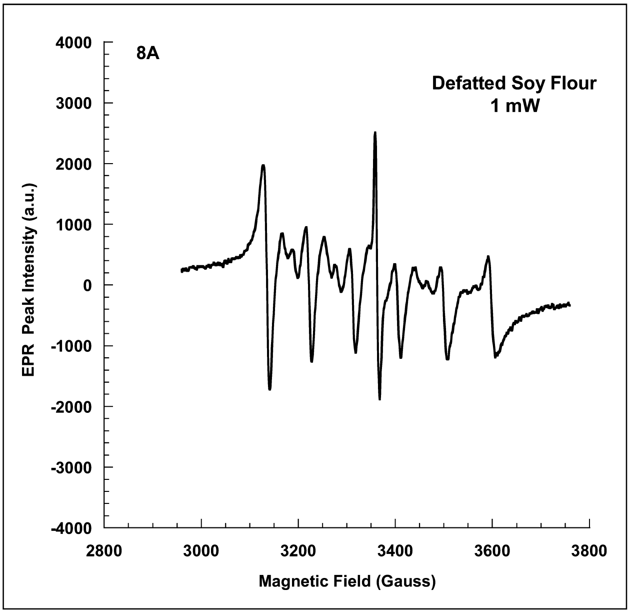


Figure 8A. Solid state EPR spectra of defatted soybean flour at 1 mW. (Reproduced from Reference (18), ©2008 John Wiley and Sons.)

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

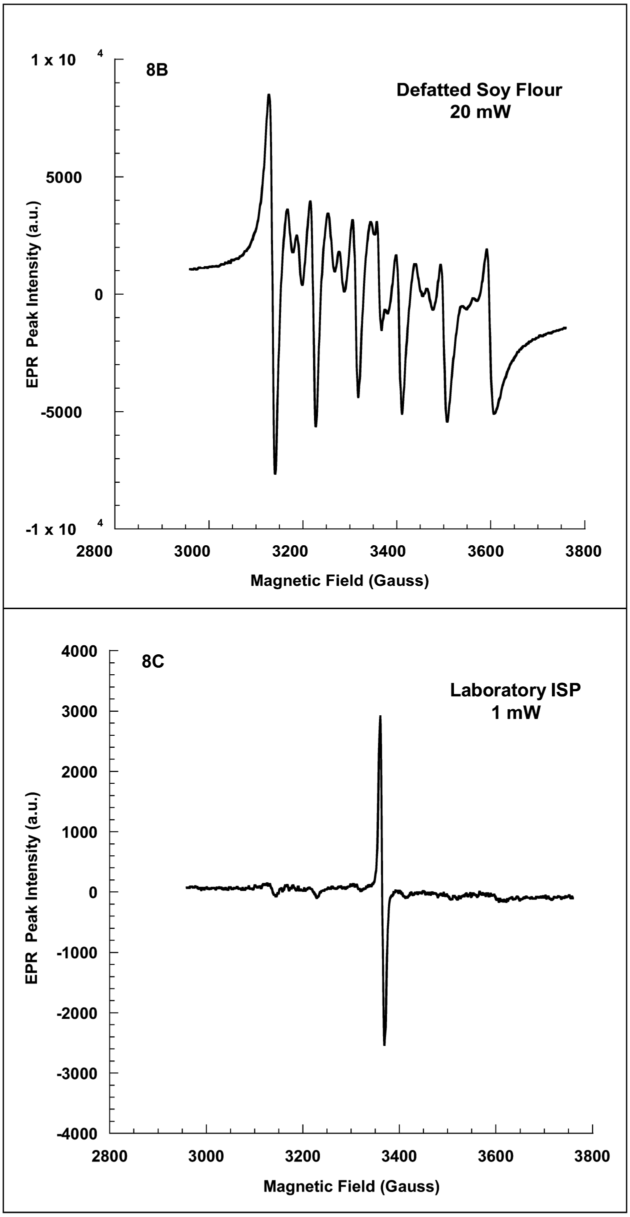


Figure 8(B-C). Solid state EPR spectra of B) defatted soybean flour at 20 mW and C) laboratory ISP at 1 mW. Reproduced from Reference (18), ©2008 John Wiley and Sons.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

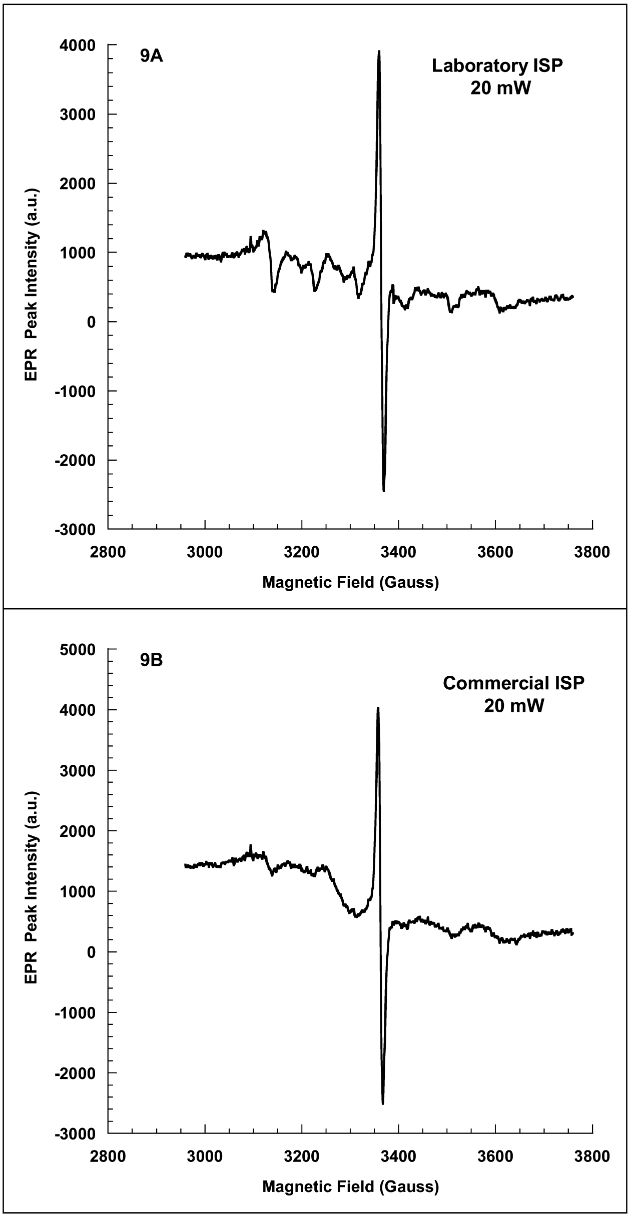


Figure 9. z. Solid state EPR spectra of A) laboratory ISP at 20 mW, and B) commercial ISP at 20 mW.

Conclusions

Concentrations of carbon-centered free radicals in ‘dry’ commercial soy protein products range from about 2.96×10^{14} to 4.10×10^{15} spins per gram, which are about 14- to 100-times greater than other food protein sources. Levels of free radicals in ‘dry’ soy protein products were found to be relatively low immediately after being produced, and then underwent large increases in free radical content during the first 10-12 weeks of storage exposed to oxygen. The radicals react to form non-radical species once the soy protein is hydrated with water. If the hydrated soy proteins are re-dried and stored in the presence of oxygen, the levels of radicals will gradually increase back to levels that existed prior to hydration. Possibly because no food proteins have previously been shown to contain such high levels of free radicals, there have been no published investigations into the deteriorative type reactions that occur once soy proteins are hydrated and these radicals are released.

Acknowledgments

The project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant numbers 2005-35503-16147 and 2009-35503-05190.

References

1. Bassetti, C.; Yu, M.-H. *Environmental Toxicology: Biological and Health Effects of Pollutants*, 2nd ed.; CRC Press: Boca Raton, FL, 2005; p 42.
2. Parsons, A. F. *An Introduction to Free Radical Chemistry*, 3rd ed.; Wiley-Blackwell: Hoboken, NJ, 2000; p 36.
3. Davies, K. J. A.; Delsignore, M. E.; Lin, S. W. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *J. Biol. Chem.* **1987**, *262*, 9902–9907.
4. Gajewski, E.; Fuciarelli, A. F.; Dizdaroglu, M. Structure of hydroxyl radical-induced DNA-protein crosslinks in calf thymus nucleohistone in vitro. *Int. J. Radiat. Biol.* **1988**, *54*, 445–459.
5. Dizdaroglu, M.; Gajewski, E.; Reddy, P.; Margolis, S. A. Structure of hydroxyl radical induced DNA-protein cross-link involving thymine and tyrosine in nucleohistone. *Biochemistry* **1989**, *28*, 3625–3628.
6. Breen, A. P.; Murphy, J. A. Reactions of oxyl radicals with DNA. *Free Radical Biol. Med.* **1995**, *18*, 1033–1077.
7. LaMarr, W. A.; Sandman, K. M.; Reeve, J. N.; Dedon, P. C. Differential effects of DNA supercoiling on radical-mediated DNA strand breaks. *Chem. Res. Toxicol.* **1997**, *10*, 1118–1122.
8. Davies, K. J. A. Protein damage and degradation by oxygen radicals. I. General aspects. *J. Biol. Chem.* **1987**, *262*, 9895–9901.
9. Neuzil, J.; Gebick, J. M.; Stocker, R. Radical-induced chain oxidation of proteins and its inhibition by chain-breaking antioxidants. *Biochem. J.* **1993**, *293*, 601–606.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

10. Khan, N.; Wilmot, C. M.; Rosen, G. M.; Demidenko, E.; Sun, J.; Joseph, J.; O'Hara, J.; Kalyanaraman, B.; Swartz, H. M. Spin traps: In vitro toxicity and stability of radical adducts. *Free Radical Biol. Med.* **2003**, *34* (11), 1473–1481.
11. Gordy, W.; Ard, W. B.; Shields, H. Microwave spectroscopy of biological substances. I. Paramagnetic resonance in x-irradiated amino acids and proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1955**, *41*, 983–996.
12. Henriksen, T.; Sanner, T.; Pihl, A. Secondary processes in proteins irradiated in the dry state. *Radiat. Res.* **1963**, *18*, 147–162.
13. Uchiyama, S.; Uchiyama, M. Free radical production in protein-rich food. *J. Food Sci.* **1979**, *44*, 1217–1220.
14. Lee, E-J.; Volkov, V. I.; Byun, M-W.; Lee, C-H. Detection of free radicals in gamma-irradiated soybean paste and model system by electron spin resonance spectroscopy. *Radiat. Phys. Chem.* **2002**, *64*, 61–66.
15. Huang, Y.; Yu, D.; Hua, Y.; Qiu, A. Detection of free radical transfer in lipoxygenase I-B-catalyzed linoleic acid-soybean protein interaction by electron spin resonance spectroscopy (ESR). *J. Agric. Food Chem.* **2006**, *54*, 9216–9220.
16. Pshezhetskii, S. Y.; Kotov, A. G.; Milinchuk, V. K.; Roginskii, V. A.; Tupikov,V. I. *EPR of Free Radicals in Radiation Chemistry*; Halsted Press: New York, 1972; pp 223−286.
17. Henriksen, T.; Meloe, T. B.; Saxeboel, G. Free radical formation in proteins and protection from radiation damage. *Free Radicals Biol.* **1976**, *2*, 213–56.
18. Boatright, W. L.; Jahan, M. S.; Walters, B. M.; Miller, A. F.; Cui, D.; Hustedt, E. J.; Lei, Q. Carbon-centered radicals in isolated soy proteins. *J. Food Sci.* **2008**, *73* (3), C222–C226.
19. Cage, B.; Weekley, A.; Brunei, L.-C.; Dalal, N. S. K_3CrO_8 in K_3NbO_8 as a proposed standard for g-factor, spin concentration, and field calibration in high-field EPR spectroscopy. *Anal. Chem.* **1999**, *71*, 1951–1957.
20. Boatright, W. L.; Lei, Q.; Jahn, M. S. Effect of storage conditions on carbon-centered radicals in soy protein products. *J. Agric. Food Chem.* **2009**, *57* (17), 7969–7973.
21. Kosuge, T.; Tsuji, K.; Wakabayashi, K.; Okamoto, T.; Sudo, K.; Iitaka, Y.; Itai, A.; Kawachi, T.; Nagao, M.; Yahagi, T.; Seino, Y. Isolation and structure studies of mutagenic principles in amino acid pyrolysates. *Chem. Pharm. Bull.* **1978**, *26*, 611–619.
22. Uyeta, M.; Kaneda, T.; Mazaki, M.; Taue, S. Studies on mutagenicity of foods. 1. Mutagenicity of food pyrolysates. *J. Food Hyg. Soc. Japan* **1978**, *19*, 216–233.
23. Nawar, W. W. Lipids. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; Marcel Dekker: New York, 1996; pp 225–320.
24. Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997**, *272* (33), 20313–20316.
25. Stadtman, E. R.; Levine, R. L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **2003**, *25*, 207–218.
26. Boatright, W. L.; Hettiarachchy, N. S. Effect of lipids on soy protein isolate solubility. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1439–1444.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

27. Boatright, W. L.; Hettiarachchy, N. S. Spray-dried soy protein isolate solubility, gelling characteristics and extractable protein as affected by antioxidants. *J. Food Sci.* **1995**, *60*, 806–809.
28. Dimmey, L. J.; Gordy, W. Electron spin resonance of 17O-labeled protein-peroxide radicals: Zein and edestin. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77* (1), 343–346.
29. Taub, I. A; Kaprielian, R. A.; Halliday, J. W.; Walker, J. E.; Angelini, P.; Merritt, C., Jr. Factors affecting radiolytic effects in foods. *Radiat. Phys. Chem.* **1979**, *14*, 639–653.
30. Priestley, D. A.; Werner, B. G.; Leopold, C. A.; McBride, M. B. Organic free radical levels in seeds and pollen: The effects of hydration and aging. *Physiol. Plant.* **1985**, *64*, 88–94.
31. Boatright, W. L.; Lu, G. Hexanal synthesis in isolated soy proteins. *J. Am. Oil Chem. Soc.* **2007**, *84* (3), 249–257.
32. Lei, Q.; Boatright, W. L. Sulfite radical anions in isolated soy proteins. *J. Food Sci.* **2007**, *72* (5), C302–307.
33. Waggle, D. H.; Kolar, D. W. Types of Soy Products. In *Soy Protein and Human Nutrition*; Academic Press: New York, 1979; p 41.
34. Buettner, G. R.; Jurkiewicz, B. A. Catalytic metals, ascorbate and free radicals: Combinations to avoid. *Radiat. Res.* **1996**, *134*, 532–541.
35. Reed, G. H.; Cohn, M. Electron paramagnetic resonance spectra of manganese (II)-protein complexes. Manganese (II)-concanavalin A. *J. Biol. Chem.* **1970**, *245*, 662–667.
36. Su, C.; Sahlin, M.; Oliw, H. Kinetics of manganese lipoxygenase with a catalytic mononuclear redox center. *J. Biol. Chem.* **2000**, *275* (25), 18830–18835.
37. Henriksen, T. Radiation induced free radicals in sulfur compounds. ESR studies in the polycrystalline state. *J. Chem. Phys.* **1962**, *37* (10), 2189–2195.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch001

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Chapter 2

Trypsin Inhibitor Activity in Laboratory-Produced and Commercial Soymilk

S. H. Yuan and Sam K. C. Chang*

Department of Cereal and Food Sciences, North Dakota State University,
NDSU Dept 2710, Fargo, ND 58108-6050
***Kow.chang@ndsu.edu**

Trypsin inhibitor activity (TIA) in soymilk processed by traditional, steam injection, blanching and UHT methods were determined and compared to commercial soymilk products. Soybeans were soaked and blanched under several selected conditions. The blanched beans were made into base soymilk. Hexanal of the base soymilk was analyzed as a soy odor marker for selecting the best conditions for further thermal processing by indirect and direct UHT methods at 135-150 C for 10-60 s using the Microthermics DIP processor. Soymilk was also made by traditional batch stove cooking and steam injection methods. TI activities of eighteen commercial products were tested for comparison. Results showed residual TIA in soymilk processed by the traditional and steam injection to 100 C for 20 min were approximately 13%. Blanching inactivated 25-50% of TI activities of the raw soymilk. The blanching conditions of 80 C and 2 min were selected for UHT processing since these conditions produced blanched soymilk without hexanal. The TI activity decreased with increased temperature and time of heating. The maximal TI inactivation was achieved by UHT direct and indirect methods with residual activities of approximately 10%. Heat inactivation (denaturation) of TIA followed first-order reaction kinetics. Some commercial soymilk products contained high TIA. TI has recently been found to have health benefits. The importance of trypsin inhibitors to the food industry and consumers was discussed.

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Introduction

Soy food consumption in the Western countries has increased dramatically due to its potential health benefits (*1*). Soy foods contain significant health-promoting bioactive components such as proteins and isoflavones but also contain undesirable beany flavor (*2*, *3*) and trypsin inhibitor activity (TIA). It is well known the consumption of the raw and inadequately cooked bean causes a decrease in protein digestibility and nutritive value and also causes pancreatic hypertrophy (*4–6*). The deleterious effect is due to trypsin inhibitors (TI) and lectins and the compact structure of the native forms of soybean major storage proteins (*4*). When trypsin inhibitors are heat inactivated, lectins, lipoxygenases and major storage proteins are also denatured. Trypsin inhibitors in soybeans consist of two types, namely the Kunitz trypsin inhibitor and the Bowman-Birk inhibitor (*7*), which also is a chymotrypsin inhibitor and is the major form of TI in cooked soymilk (*8*). Instead of protein unfolding during heat denaturation, interchanges of disulfide linkages (cystine residues) between inhibitors and storage proteins such as glycinins, and the degradation of cysteine/cystine have been hypothesized to be partly responsible for the inactivation of Bowman-Birk trypsin inhibitor (*7*).

Hackler et al. (*9*) reported that 4-10% residual TIA in soymilk gave the highest protein nutritive value for the heated soymilk. However, inactivating 100% trypsin inhibitor may cause overheating, which damages soy proteins by destroying lysine, tryptophan and cysteine in the soymilk (*9–11*). Cysteine retention is very important to protein nutritive value since it is one of the two sulfur amino acids that are the limiting essential amino acids in soy foods. Cysteine destruction also has been related to off-flavor (*11*). Research discoveries from the last two decades have shown purified Bowman-Birk inhibitor has protective effect against oral and other cancers (*12–15*). However, it is not known if the low concentrations that are present in heated soy foods can have any beneficial effect in humans. Bowman-Birk inhibitor can stimulate the secretion of trypsin, chymotrypsin and carboxypeptidases and cause an increase in cholescystokinin in humans (*16*). Therefore, caution must be taken in the interpretation of the long-term impact to the frequent users of high levels of Bowman-Birk inhibitors.

Kwok and others (*17–19*) investigated the effect of indirect UHT heating on soymilk trypsin inhibitors by placing soymilk, in small-diameter stainless tubes, which were heated in an oil bath at selected temperatures. They reported that heating at 143 C for approximately 60 s can inactivate TI to approximately 10% of the original raw soymilk. Under some conditions, UHT may achieve commercial sterility, but the heat may not achieve adequate inactivation of trypsin inhibitors in the final products as some commercial products have been found to be much higher than 10% TI. A study (*20*) conducted in Hong Kong, several UHT-processed commercial soymilk products contained very high trypsin inhibitor activities (20-47% of the TI of raw beans). Liener (*21*) expressed a concern of consuming trypsin inhibitor residues in the range of 5-20% of the raw bean may have negative effect on human health. Ensuring a low trypsin inhibitory activity is important to health, particularly to infants or young children who can not drink cow's milk and rely on soy formula or soymilk as the primary source of protein for growth. The long-term consumption of high levels of residual trypsin inhibitors also may affect the health of frequent soy-consuming vegetarians. It is important that trypsin inhibitory activity be considered in designing thermal processes to produce the lowest beany off-flavor intensity and to retain the highest nutritional profiles. Although the kinetic analyses of trypsin inhibitor inactivation using indirect UHT heating and hydrothermal steam fusion have been reported (*8*, *18*, *19*, *22*, *23*). Their studies have not used a continuous vacuum process such as that used in the direct-steam injection process by the Microthermics DIP processor, and have not used a pre-treatment to reduce odor production of the soymilk. Ultra-high temperature (UHT) is a relatively new processing method for processing soymilk in modern soymilk manufacturing companies. The small pilot-scale of the Microthermics DIP Processor mimics modern commercial UHT processes for soymilk processing. Another factor affecting trypsin inhibitor activity is material differences due to variety (genotype). Accordingly, we have completed the research with the objective to determine TIA in soymilk made from two soybean varieties and processed by traditional indirect and steam injection, and to determine the effect of soybean blanching and UHT processing methods using the Microthermics DIP processor on the inactivation of trypsin inhibitors (*24*).

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Materials and Methods

Soybean Materials

Soybeans (*Glycine max*) of the variety of Proto (harvested in 2005 and 2006) were obtained from Sinner Brothers and Breshnahan (Casselton, ND). IA2032, a lipoxygenase-null variety (harvested in 2005), was obtained from Stonebridge Ltd. (Cedar Falls, Iowa). Commercial soymilk products of various brands produced in 2006 were obtained from the local grocery stores in Fargo, North Dakota. The brands included Better-Than-Milk and Shaklee powder products, and 8th Continent, Soy Dream, Soy Slender, West Soy, and WhiteWave Silk liquid products.

Soymilk Processed by Traditional and Steam-Injection Batch Processes

Whole Proto (2005) and IA2032 soybeans were soaked in tap water at the room temperature for 15 h. The soaked beans were drained, rinsed, and ground with tap water using to the bean:water ratio of 1:9 (w/w). In the traditional (atmospheric pressure) batch cooking treatment, soybeans were ground for 3 min at the high speed using Hamilton Beach blender (model: 585-1, Peabody, MA). The soy slurry was filtered through a muslin cloth to separate the insoluble residues from the soymilk. The raw soymilk (1 L) in a small pot was heated within a larger pot, which contained boiling water on a stove, which was set at the highest heat level, to approximately 90C, and then the small soymilk pot was switched to the hot stove surface to heat to 100C and held at this temperature with stirring to prevent foaming for up to 30 min. It took approximately 8 min of heating to bring the temperature to boiling. Soymilk (approximately 30 mL) was sampled at 0, 3, 6, 9, 12, 20 and 30 min after boiling. Immediately after sampling at each time interval, the soymilk in a small beaker was cooled in an ice bath. The soymilk was freeze-dried and analyzed for TI activity.

For the direct steam injection treatment, soymilk (2 L) was produced by a continuous grinder equipped with an auto-centrifugal separator with 120-mesh size screen (Chang-Sheng Machinery Co., Taoyuan city, Taiwan). The soymilk came out of the continuous grinder was immediately injected with live steam at about 45 psi to boiling and held for 20 min at boiling. It took approximately 15 s for soymilk to reach boiling. Soymilk was sampled at 0, 3, 6, 9, 12 and 20 min after boiling. Immediately after sampling at each time interval, the soymilk in a small beaker was cooled in an ice bath. The soymilk was freeze-dried and analyzed for TI activity.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Soymilk Processed by Blanching and UHT Methods

The following processing methods were carried out.

A. Selected literature UHT methods

(1) **The processing conditions of 143 C for 60 s reported by Kwok and others** (*17–19*): Approximately 2 kg of Proto (2005) and IA2032 soybeans were soaked for 15 h at room temperature. The soaked soybeans were drained and ground in water with a 9:1 water-to-bean ratio using an automatic centrifugal grinder (Chang-Seng (Machinery, Taiwan). The soymilk was processed using the Microthermics UHT/HTST DIP Processor (Raleigh, NC, USA) by two methods: the indirect and the direct steam injection-vacuum cooling methods. The Microthermics Processor heated the soymilk in two stages. The first stage was pre-heating, which was set at 110 °C. The flow rate was set at 1 L/min. The second stage was heated by either heat exchanger in the indirect mode or by direct steam injection in the direct mode to 143C. The heated soymilk was pumped through a well-insulated holding tube (60 s) so that the processing could be continued in a continuous manner. The tube length was constructed in a way to allow the soymilk to flow through with a desired time at the flow rate of 1 mL/min. In addition to the build-in insulation, which covered the tubes, the entire holding tube coil set was further covered with a heavy blanket to maintain the holding temperature. In the indirect mode, the soymilk came out of the holding tube was cooled by a tubular heat exchanger using cold tap water. In the direct steam-injection mode, a vacuum cooling chamber was used to remove the water condensed from the steam injected and to remove odor. The temperature of the product at the vacuum chamber was set at 110 C to maintain the same solid content as that of the raw soymilk. After vacuum cooling, the product was

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

further cooled to 23 C by circulating tap water in a tubular heat exchanger.

(2) **Commercial UHT settings reported by Prabhakaran and Perera** (*25*): The 2-stage processing method was reported to be a common commercial practice in Singapore. The first stage was 120 C for a longer period of time (80 s) to inactivate 80% TI, and the second stage was 140 C for a short period of time (4-6 s) to take advantage of the high power of sterilization before aseptic packaging. Therefore, a 2-stage process using our Microthermics Processor was carried out to mimic this commercial practice to test its effect on TI inactivation. Proto and IA2032 were processed into raw soymilk using the methods described in the traditional cooking methods as described above. The raw soymilk was then processed at 120C for 80 s, cooled to the room temperature, and followed by direct and indirect UHT heating at 140 C for 4 s (with on-line pre-heating at 110C). The soymilk obtained was freeze-dried and analyzed for TI activity.

B. Blanching of soaked soybeans followed by UHT inactivation of TI

(1) **Blanching experiments**:
For minimizing the soy odor generation during soymilk manufacturing, experiments were carried out to inactivate the lipoxygenase activities of Proto soybean since it contained high lipoxygenase activity (*2*). Soybeans (Proto of the 2006 crop) were soaked for 15 hr at the room temperature (~22C). The soaked soybeans were rinsed with the tap water and immersed for a period of time ranging from 0.5 to 10 min in a large tank of hot water (140 L), which was maintained manually from 70 to 85C using a live steam injector. The heated soybeans were cooled in a tank containing cold water (10-15 C) immediately after heating. The cooled soybeans were made into soymilk using the automatic soymilk machine as described above for traditional steam injection method. The soymilk obtained was analyzed for mass yield (g soymilk produced per 100g soybean), protein content, and % protein recovery (extraction rate) as compared to the raw soymilk made from unblanched soaked soybeans. The TI activity and hexanal in the soymilk were analyzed. The lipoxygenase activity of the soymilk with the lowest hexanal content was also analyzed to ensure the enzyme was totally inactivated by the heating conditions.

(2) **Direct-UHT processing of the soymilk made from blanched soybeans**:
One Kg Proto soybeans (2006 crop) were soaked at the room temperature for 15 hr to rehydrate, and blanched in 140 L hot water at 80C for 2 min. The heated beans were cooled in a

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

tank of cold tap water (140 L). The cooled blanched soybeans were processed into soymilk, preheated at 110C using the Microthermics DIP processor, which then heated the soymilk with a continuous flow at 135, 140, 145 and 150 C for 10 to 50 sec, respectively, using the direct steam-injection mode, which was equipped with a vacuum chamber for removing water and soy odor. The reason for cooling the soybeans immediately after blanching was to control the heat remained in the whole soybeans in between processing steps since in our laboratory was not equipped with a continuous transport system between blancher and grinder for extraction of soymilk from the whole blanched beans, and in between extracted soymilk from the exit of the grinder and the UHT heat processor. Such continuous integrated transport/heating systems exist in modern commercial practice to eliminate the effect of transit time.

(3) **Indirect UHT processing of soymilk made from blanched soybeans**:

Soymilk was processed from Proto soybeans in the same manner as described in the section **(2)** of the direct method and preheated at 110C using the Microthermics DIP processor, which then heated the soymilk with a continuous flow at 135, 140, 145 and 150 C for 20 to 30 sec, respectively, using indirect heat exchange tubes, which did not contain a vacuum chamber for removing water and soy odor. Maximum time of 30 sec was chosen since longer than 30 sec, the soymilk heated by the indirect method formed precipitates.

Lethality (F_0) was calculated according to the equation $Fo=10^{(T-121)/Z}$ for comparing the heat power for inactivating bacteria spores or TI. When comparing to the inactivation of bacterial spores PA 3679 (*Clostridium sporogenes*) in non-acidic foods such as soymilk, the Z value of 10 was applied (*26*). When testing the heat power for the inactivation of TI, the Z value of 28 as proposed by Kwok and others (*17*) was applied.

Chemical Analyses

1. **Trypsin inhibitor activity analysis**: The trypsin inhibitor activity assay of Kakade and others (*27*) was used. The TI activity was expressed as TIU per g soymilk on a dry basis. TI activity also was expressed as mg trypsin inhibitor equivalent by dividing the TIU by 1900 since each mg trypsin inhibitor produced 1900 TI units (*28*).
2. **Soy odor using hexanal as a marker**: Solid phase microextraction (SPME) method was used to extract the volatile compounds in the soymilk. The extracted odors were analyzed by gas chromatography. The details of external and internal standards, SPME and GC conditions were according to Yuan and Chang (*2*, *3*). Results were expressed as ppm (µg/mL soymilk).

3. **Lipoxygenase activity**: The lipoxygenase activity of the soymilk was analyzed according to the method of Anthon and Barrett (*29*).
4. **Protein content**: Protein in soymilk and soybean samples was determined by the Kjeldhal Method 988.05 of AOAC Intl. (*30*) using a protein conversion factor of 6.25.

Kinetic Analysis of Trypsin Inhibitor Activity

Kinetic analysis of residual trypsin inhibitor activity in soymilk processed by direct UHT processing method as described in the above experiments was carried out according to the combined model the two trypsin inhibitors in soymilk: Kunitz soybean trypsin inhibitor (KSTI) and Bowman-Birk inhibitor (BBI) as reported by Rouhana and others (*8*). Such mixed activity model was also used by Kwok and others (*19*) in their mathematical analysis of TI inactivation. The inactivation of TI activity at a give time (t) can be expressed in the following equation (*24*):

$\ln N_t = -kt + \ln N_{0,}$ where N_t and N_0 are the TI activity at time t and 0, respectively.

The inactivation rate constant (k) at each specific temperature from 135 to 150 C, respectively, was calculated by plotting the ‘natural log’ (ln) of the residual TIA versus time (s). Further, lnk values at each temperature of inactivation were plotted against (1/T, reciprocal of absolute temperature, °K) according to the Arrhenius equation of

$$\ln k = \ln k_0 - E_a/RT$$

The slope of this equation was -Ea/R, where R is the gas constant, -8.314 J mole^{-1} $°\text{K}^{-1}$. Therefore, the Ea was equal to the slope of the negative line multiplying by the R value (*8*).

Trypsin Inhibitor Activity of Commercial Soymilk

Eighteen commercial soymilk products (marketed in 2006) were purchased from local grocery stores. The TI activities of these products were analyzed using the method described above.

Statistical Analysis

Soymilk production was completed in duplicate, and each sample at each processing method-time was analyzed twice. The data were subjected to the analysis of variance using the SAS 9.1 package (*31*). Significant differences among treatments were analyzed using Duncan’s Multiple Range test with probability level of < 0.05.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Results and Discussion

Trypsin Inhibitor Activity As Affected by Traditional and Steam-Injection Methods

Table I shows the trypsin inhibitor activities in two food soybean cultivars as affected by the traditional and steam injection methods (atmospheric heating conditions) for up to 20-30 min. The raw soymilk made from IA2032 possessed a similar TI activity as compared to that made from Proto soybean. When the soymilk was heated from raw to boiling at 100C by the traditional cooking method, the residual TI activity ranged from 55-66%, which was similar to that (57%) reported by Miagi and others (*32*). Steam injection seemed to have a higher power in inactivating TI in Proto soymilk than that in IA2032 soymilk. After boiling by traditional cooking or steam injection for 9 min, more than 20% of the original TI activities still remained. When soymilk was boiled at 100 C for 20 min, 82-87% of the TI activities were inactivated, whereas Miagi and others (*32*) reported 95% trypsin inhibitors were inactivated (5% residual activity) under the same boiling temperature and time. Boiling soymilk by the traditional cooking method at 100C for 30 min was necessary to reduce TI activities to about 10% or less. This heat requirement was greater than the 10 min at 100C reported by Miyagi and others (*32*), similar to that (29 min at 99C) observed by Johnson and others (*22*), but was less than that (60 min at 99C) reported by Wallace and others (*33*). The differences between our study and others were not clear; however, may be due to differences in soybean materials and experimental conditions such as different water-to-bean ratios, processing devices and heating rates. The inactivation curves (not shown) of the two atmospheric heating methods did not fit the first-order reaction kinetics since ln residual activities versus time was not a linear line.

Table I. Trypsin Inhibitor Activity as Affected by Traditional and Steam Injection Methods under Atmospheric Pressure for Various Time Periods

Heating time (min)	*TIU/g*		*mg TI/g*		*Residue%*	
	Proto	*IA2032*	*Proto*	*IA2032*	*Proto*	*IA2032*
Traditional method (100C)						
Raw	66470	72750	35.0A	38.3A	100	100
0	44090	39970	23.2B	21.1B	66.5	55.1
3	26700	22360	14.1C	11.8C	40.3	30.8
6	18880	19720	10.0D	10.4DC	28.5	27.1
9	13600	16850	7.2E	8.9DE	20.5	23.2
12	11140	14600	5.9EF	7.7E	16.8	20.1
20	8870	9910	4.7Fb	5.3Fc	13.4	13.6

Continued on next page.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Table I. (Continued). Trypsin Inhibitor Activity as Affected by Traditional and Steam Injection Methods under Atmospheric Pressure for Various Time Periods

Heating time (min)	*TIU/g*		*mg TI/g*		*Residue%*	
	Proto	*IA2032*	*Proto*	*IA2032*	*Proto*	*IA2032*
30	5130	7770	2.7G	4.1F	7.7	10.7
Steam Injection (100C)						
Raw	65200	63200	34.3A	33.3A	100	100
0	26960	38910	14.2B	20.5B	41.4	61.6
3	19230	27170	10.2C	14.3C	29.5	43.0
6	15820	22240	8.3D	11.8D	24.3	35.2
9	13350	18790	7.0E	9.9E	20.5	29.8
12	12900	17230	6.8E	9.1F	19.8	27.3
20	8429	11700	4.5Fb	6.2Gb	12.9	18.2
UHT (143C, 60s)						
Raw	65200	63200	34.3A	33.3A	100	100
Direct	13510	12740	7.2Ba	6.7Ba	20.7	20.2
Indirect	13650	10320	7.1Ba	5.4Cc	21.0	16.3

[*] Data are means of two replicates. Means with different capital letters A, B, C…in the same column indicate significant differences among different heating times (raw, 0, 3, 6…….) of mg TI/g at $p<0.05$. [*] Means with different small letters, a, b, c, d among four processing methods (traditional method 20 min, steam injection 20min, direct and indirect UHT at 143C, 60 s) are significant at $p<0.05$. Source: Reference (*24*), Copyright ACS.

Trypsin Inhibitor Activity As Affected by Selected UHT Methods

Kwok and others (*18*, *19*) reported that indirect batch UHT heating conditions (143 C for 60 s in a capillary tube immersing in an oil bath) could reduce the TI activities to less than 10% of the original activity. We decided to analyze the TI activity in the soymilk heated by similar conditions (143 C for 60 s using a holding tube that was made to be 60 s length at a 1L/min flow rate) to confirm if that was true for our soymilk heated by a continuous Microthermics Processor. We also compared these processing conditions to that practiced in the commercial settings (120 C for 80 s +140 C for 4 s). In addition to this process that integrated these two temperature-time procedures, we also tested the characteristics of heat inactivation by 120C for 80 s and 140C for 4 s, separately, to test the TI inactivation at each respective process. Table II shows the results of TI inactivation by these selected UHT processing methods. The results indicated that 16-21% of the TI still remained after indirect UHT heating at 143 C for 60 s, while approximately 20%

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

remained after direct UHT heating. The discrepancies in TI inactivation by similar processing temperature-time combinations between our results and that of Kwok and others (*17*) might be due to differences in processing methods and soybean materials. It is interesting to note that 120C, 80 s produced similar TI inactivation effect as the 140C, 4 s process. Integrating these two processes did not increase much TI inactivation, particularly with no improvement for IA2032 soymilk in the indirect heating mode.

Table II. Trypsin Inhibitor Activities in Soymilk made from Proto and IA2032 by Selected UHT Processing Methods

		TIU/g		*mg TI/g*		*Residue%*	
Heating time (min)	*F_0Value*	*Proto 05*	*IA2032*	*Proto 05*	*IA2032*	*Proto 05*	*IA2032*
UHT Indirect							
Raw		65200	63200	34.3A	33.3A	100	100
143C, 60s	6.11	13650	10320	7.1C	5.4D	21.0	16.3
120C, 80s	1.23	16890	17490	8.9B	9.2B	25.9	27.7
140C, 4s	0.32	15350	15350	8.1B	8.1C	23.5	24.3
120C, 80s + 140C, 4s	1.55	13110	15260	6.9C	8.0C	20.1	24.1
UHT Direct							
Raw		65200	63200	34.3A	33.3A	100	100
143C, 60s	6.11	13510	12740	7.1B	6.7B	20.7	20.2
120C, 80s	1.23	12560	11870	6.6C	6.2C	19.3	18.8
120C, 80s + 140C, 4s	1.55	9840	9310	5.2D	4.9D	15.1	14.7

[*] Data are means of two replicates. Means with different capital letters A, B, C…in the same column indicate significant differences among different heating treatments within each of the direct or indirect methods at $p<0.05$. Lethality (F_0) was calculated according to the equation $F_0=10^{(T-121)/Z}$, where Z is 28 C for inactivating TI in soymilk. Source: Reference (*24*), Copyright ACS.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

When lethality (F_0) values (Table II) among these selected processing conditions were compared using the Z= 28C as reported by Kwok and others (*17*), we found that the differences between residual TI among these processes did not behave according to the calcuated lethality power manner since F_0 was 6.1 for the 143C, 60 s conditions and 0.32 for the 140C, 4 s conditions (the ratio was about 19 or 1900%), but the residual TI did not change that much (only 2 to 8% differences). Therefore, the mathematical model that published by Kwok and others (*17*) using a batch heating system could not be applied to our laboratory conditions. This is interesting and implies that the kinetics of TI inactivation must be developed for each respective industrial processing condition, because processing variables such as grinding method, solid content, pretreatment method, machinery design, and final heating method differed in various soymilk manufacturers. For this reason, we conducted further experiments to test the kinetics of TI inactivation in conjunction with our intent to improve soy flavor by first inactivating lipoxygenases in soybeans with blanching prior to the UHT processing.

As compared to the UHT direct and indirect heating at 143 C for 60 s, traditional atmospheric cooking and steam injection at 100C for 9 min was required to produce similar TI inactivation for Proto soymilk whereas it took 12-20 min at 100C for IA2032 soymilk to produce similar effect (Table I). It should be noted that the lethality at 100C for 9 to 20 min was minimal as compared to that at 143C for 60s.

Soybean materials may contain different proportions of Kunitz and Bowman-Birk inhibitors, and processing methods, including water-to-bean ratio which could affect the extraction and heat stability of two different types of trypsin inhibitors in soybeans (*7*, *8*). Heating could cause not only the denaturation of proteins due to changes in protein secondary and tertiary structures, but also may cause interchanges of disulfide bonds between different proteins (*7*) to cause aggregations. In addition, under severe conditions, heating soymilk may degrade amino acids such as cysteine (*10*, *11*) and lysine (*9*) indicating potential Maillard reactions between lysine and reducing sugars. Unlike purified TI in water experimental model system, trypsin inhibitors in natural complex soymilk food systems may interact with other proteins such as glycinins and reducing sugars during thermal processing. Such interactions may affect TI activities. A recent report that UHT processing at 142C for 4-6 s could eliminate the ability of soy protein to decrease LDL-cholesterol in humans (*34*). The authors attributed the changes in cholesterol lowering effect to protein structural alterations by ultra heat treatment.

Effect of Blanching

The results of blanching whole soybeans on soymilk yield and protein extraction in terms of % protein in soymilk and total protein recovery in soymilk are presented in Table III. Soymilk yield was not largely affected due to a constant water-to-bean ratio used for extraction. There was a trend within each temperature that protein recovery was slightly decreased with the increase in heating time.

Table III. Blanching Effect on Soymilk Yield and Protein Recovery*

Temperature (C)	*Time (min)*	*Soymilk Yield (g/100 g bean)*	*Protein (%)*	*Protein recovery (%)*
Raw		806	3.53	100
70	5	823	3.25	94
70	7.5	820	3.24	93
70	10	829	3.13	91
75	2	823	3.26	94
75	3	824	3.19	92
75	4	832	3.16	92
80	0.5	816	3.27	94
80	1	792	3.26	91
80	2	800	3.18	89
80	3	790	3.16	88
85	0.5	801	3.19	90
85	1	800	3.25	91
85	1.5	775	3.13	85

* Data are means of two replicates. Source: Reference (*24*), Copyright ACS

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

The effect of blanching on TI inactivation and hexanal generation in soymilk are presented in Table IV. The residual TI in the soymilk prepared from blanched soybeans ranged from 51% when heated at 80C for 3 min to approximately 75% when heated at 80C or 85C for 30 s. Please note that the TI in the raw soymilk made from Proto was higher than that presented in Table I. This was due to the Proto materials were from different years of harvest. The Proto used for Table I and Table II was harvested in 2005, whereas the Proto used for blanching experiments was from 2006 crop. Earlier we also observed a very high TI in the raw Proto soymilk in our yuba research (*35*). Therefore, the year of production presented a seasonal effect on the soybean chemistry. The hexanal content in the soymilk made from blanched soybeans under all conditions were all largely decreased, and with no hexanal detected in soymilk processed by 80C for 2 min at which the protein recovery was 89%. The lipoxygenase test showed that the enzymes were inactivated under these conditions. Therefore, the conditions of 80C for 2 min were chosen for further UHT heating experiments due to the consideration for enhancing flavor in the soymilk products. Wilkens and others (*36*) reported soymilk produced by hot grinding of soybeans at approximately 80C or higher could lower soy odor concentrations in soymilk products. Endo and others (*37*) reported heating whole soybeans on hydroperoxide reduction in normal soybean. They reported under some steaming and boiling conditions, soybeans could produce soymilk with similar levels of hydroperoxides as that prepared from lipoxygenase-null soybeans. Sensory evaluation showed that a dessert product prepared from soymilk that was made from heat-treated soybean gave lower beany flavor scores than that made from soybean without preheating treatment. The low hexanal content in soymilk made from blanched soybeans was consistent with the low-hydroperoxide findings by Endo and others (*37*).

Table IV. Trypsin Inhibitor Activity and Hexanal in Soymilk made from Blanched Soybeans

Blanched conditions		*TIU/g*	*TIA/g protein*	*Residual TI % of raw soymilk*	*Hexanal (ppm)*
Raw		112400A	213000	100	3.48
70C	5 min	76010CD	161800	67.6	0.52
70C	7.5 min	67760EF	138400	60.3	0.44
70C	10 min	57000G	121700	50.7	0.28
75C	2 min	72720DE	155300	64.7	0.05
75C	3 min	65360F	146700	58.2	0.09
75C	4 min	64770F	140000	57.6	0.13
80C	30 s	84900B	189000	75.5	0.76
80C	1 min	79350BC	164300	70.6	0.04
80C	2 min	64110F	134100	57.0	nd
80C	3 min	57300G	119000	51.0	0.02
85C	30 s	84650B	176500	75.3	0.54
85C	60 s	68610EF	141200	61.0	0.04
85C	90 s	65310F	137700	58.1	0.31

Data are means of two replicates. Source: Reference (*24*), Copyright ACS.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Direct UHT Processing of Soymilk Made from Blanched Soybeans

Table V shows the results of TI inactivation by direct UHT Processing of soymilk at 135-150C for 10-50s, respectively. Similar to the trends reported in the literature, TI decreased with the increase in the time of heating at each temperature, and TI decreased when temperature was increased. The only conditions that decreased the TI to about 10% of that in the raw soymilk was at 150C for 50s which gave an equivalent F_0 value of 661 for inactivation of PA3679. Johnson and others (*22*) reported a much higher heat requirement at 154C for 40 s (F_0 of 1330) for inactivating TI to less than 10% of the original TI in raw soymilk. We did not calculate F_0 for TI inactivation according to Z=28 as reported by Kwok and others (*17*) since this Z value was specific to laboratory processing conditions, and therefore, could not be applied to our experimental conditions. As shown in Table V, the behavior of TI is much more resistant than bacterial spores since TI inactivation was much lower than bacterial inactivation.

Figure 1 shows the kinetics analysis of the results of TI inactivation by the UHT processing conditions as presented in Table V. Linear lines of ln residual TI vs. time had a negative slope with correlation coefficients ranging from 0.92 to 0.95 indicating a good fit of the first-order reactions. Similar to the report of Rouhana and others (*8*), the combination model of KSTI-BBI inhibitors worked well for these sets of results from the specific conditions of this study. However, Johnson and others (*22*, *23*) reported a non-linear kinetics for soymilk TI inactivation by hydrothermal heating of soymilk. They obtained a fast inactivation period which was followed by a slow inactivation period. They attributed the second slow period of the TI inactivation to the Bowman-Birk inhibitors.

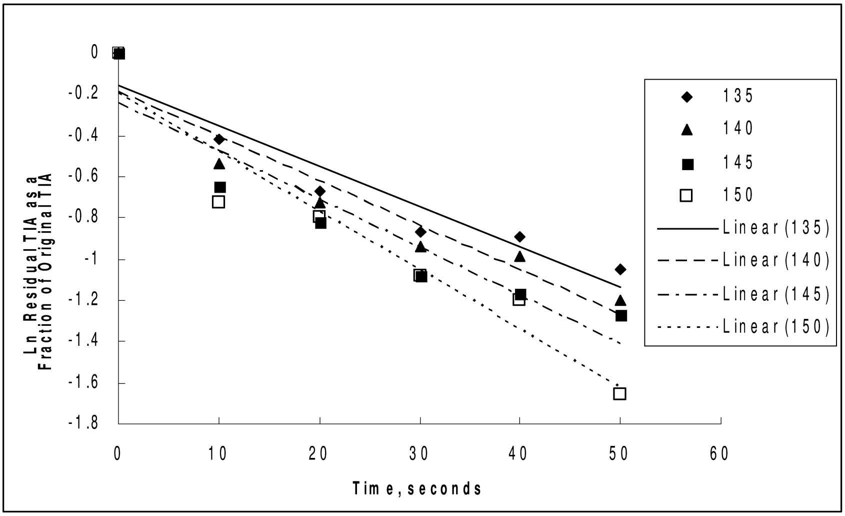


Figure 1. Thermal inactivation curves plotted using a combined inactivation model of two trypsin inhibitors in soymilk processed at 135-150 C for 10-50 s. Source: Reference (24), Copyright ACS.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Table V. Trypsin Inhibitor Activity in Soymilk Processed by Direct-UHT Processing Methods from Soybeans Blanched at 80C for 2 min

Heating conditions	*F_0* (Z=10C)*	*TIU/g*	*Residual TI% of blanched soymilk*	*Residual TI% of raw soymilk*
80C 2min		64100A	100.0	57.0
135C 10s	4.2	42400B	66.1	37.7
135C 20s	8.4	32480DE	50.7	28.9
135C 30s	12.5	27170GFH	42.4	24.2
135C 40s	16.7	26160FGHI	40.8	23.3
135C 50s	20.9	22720KIJ	35.4	20.2
140C 10s	13.2	37330C	58.2	33.2
140C 20s	26.5	30430DEF	47.5	27.1
140C 30s	39.7	24760HIG	38.6	22.0
140C 40s	52.9	23900HIJ	37.3	21.3
140C 50s	66.2	19380KL	30.2	17.2
145C 10s	41.9	33010D	51.5	29.4
145C 20s	83.8	28440EFG	44.4	25.3
145C 30s	125	22000IJKL	34.3	19.6
145C 40s	167	19830JKL	30.9	17.6
145C 50s	209	17770L	27.7	15.8
150C 10s	132	30440DEF	47.5	27.1
150C 20s	264	28560EFG	44.6	25.4
150C 30s	397	21980IJKL	34.3	19.6
150C 40s	529	19520JKL	30.4	17.4
150C 50s	661	12310M	19.2	10.9

* Lethality (F_0) was calculated according to the equation $F_0 = 10^{(T-121)/Z}$, where Z is 10 C for inactivating bacterial spores PA 3679 in non-acidic foods. Data are means of two replicates. Means followed by different letters are significantly different at $p < 0.05$. Source: Reference (*24*), Copyright ACS.

The first-order reaction constant k values for these lines obtained from Figure 1 were plotted against 1/T (reverse of temperature in °K scale) and the results are shown in Figure 2. Through linear regression (r^2 =0.95, P <0.05), the slope of the line was -4098 °K. The activation energy Ea of 34 KJ/mole was obtained by multiplying this negative slope with -8.314 J $mole^{-1}$ $°K^{-1}$. Rouhana and others (*8*) applied the combination model for calculation of the Ea of TI inactivation in soymilk and they obtained the -6670 °K, which was equivalent to Ea of about 55 KJ/mole. Based on a separate KSTI and BBK model, Rouhana and others (*8*) reported that Ea for KSTI and BBK were 24 and 102 KJ/mole, respectively. On the

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

other hand, Johnson and others (*23*) analyzed the TI inactivation of KSTI and BBK and reported 47 KJ/mole for KSTI and 20 KJ/mole for BBI. One major difference between the experiments of soymilk between Rouhana and others (*8*) and Johnson and others (*23*) was the ratios of water-to-bean materials. A water-to-bean ratio of 4 to 1 (w/w) was used by Johnson and others (*23*), whereas 10 to 1 was used by Rouhana and coworkers (*8*). The different ratios might have resulted in different extraction yields of KSTI and BBI into the soymilk. Moreover, solid and protein concentration of soymilk might have a major impact on the heat resistance of these inhibitors. This is a plausible explanation since Dipietro and Liener (*7*) discovered that heating soy flour *in situ*, where solid/protein concentration was much higher than soymilk, resulted in a faster inactivation of BBI than KSTI. However, in a diluted soy extract in water such as soymilk, BBI was much more resistant than KSTI. A low water-to-bean ratio could result in high protein concentration to promote a faster rate of BBI inactivation. Dipietro and Liener (*7*) attributed this effect to inter-disulfide bond exchanges between BBI and glycinin, a major storage protein rich in disulfide and sulfhydryl groups. When solid is high, the interactions between proteins such as inhibitors and other constituents also would be more complex to contribute to different behaviors of inhibitor inactivation as reported by various researchers.

Indirect UHT Processing of Soymilk Made from Blanched Soybeans

Table VI shows the TIA results from selected indirect UHT heating conditions. Comparing to the respective direct UHT heating conditions, indirect heating decreased TIA to a larger extent. This may be due to heat transfer across the heat exchange tubing that is needed to reach the cold point. Therefore, the regions inside the heat exchange tubing that were closer to the tube's internal surface would have exposed to a higher temperature.

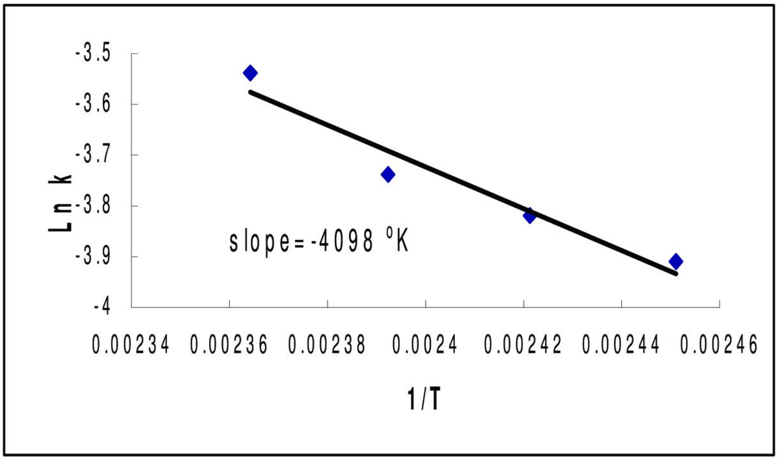


Figure 2. Arrhenius plot for the inactivation of trypsin inhibitors under ultra-high temperature conditions by direct the Microthermics UHT Processor. Source: Reference (24), Copyright ACS.

Table VI. Trypsin Inhibitor Activity in Soymilk Processed by Indirect-UHT Processing Methods from Soybeans Blanched at 80C for 2 min

Heating conditions	*F_0* (Z=10C)*	*TIU/g*	*Residual TI% of blanched soymilk*	*Residual TI% of raw soymilk*
80C 2 min		64110A	100.0	57.0
135C 20s	8.4	24820B	38.7	20.7
135C 30s	12.5	20850C	32.5	17.4
140C 20s	26.5	19390CD	30.2	16.2
140C 30s	39.7	17870DE	27.9	14.9
145C 20s	83.8	17660DE	27.5	14.7
145C 30s	125	16840E	26.3	14.1
150C 20s	264	13770F	21.5	11.5
150C 30s	397	11810G	18.4	9.9
150C 50s	661	12310M	19.2	10.9

* Lethality (F_0) was defined as in Table V. Data are means of two replicates. Means followed by different letters are significantly different at $p < 0.05$.

TIA of Commercial Soymilk Products

Table VII shows the TIA results of 18 selected commercial soymilk or soy powder samples. Some of the samples, which were numbered as 1 and 2 of the same products, were obtained and analyzed six months apart. The range of the TIA of commercial samples was large. Some of the samples had high TIA. This is a concern since high TIA content could bring deleterious effect to health of the frequent users (*21*). Soymilk can be commercially sterilized ($F_0 \geq 3$) (*20*), aseptically packaged and safely stored at ambient temperature even if 90% of TIA is not inactivated. The refrigerated soymilk products that are packaged in milk cartons should also be heated to inactivate 80-90% of TIA to reduce the anti-nutrient effect of the inhibitors. However, some soymilk manufacturers have ignored this since the trypsin inhibitor levels in commercial products have been found to be much higher than this level. Guo and others (*20*) reported the trypsin inhibitor activity of ten commercial soymilk/tofu products in Hongkong and observed unacceptably high TI activities (about 30 to 48% of the original TI) existed in most of the UHT processed soymilk and in one 100 C pasteurized soymilk. We also found that some commercial soymilk produced in the United States contained very high trypsin inhibitor activities (Table VII). The exact nutritional significance of long-term consumption of these very high trypsin inhibitor activities in the diet is unknown.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Table VII. Trypsin Inhibitor Activity in Commercial Soymilk Products*

Sample	*TIU/g*
Company A-Plain 1	16870G
Company A-Plain 2	23940E
Company A-Vanilla 1	27400D
Company A-Vanilla 2	19910F
Company A-Unsweetened 1	34900B
Company A-Unsweetened 2	39740A
Company A-Plain-Lactose Free	15240H
Company B	23000E
Company C-Low Fat	5660K
Company C-Non-Fat	6290KJ
Company C-Plain	12580I
Company D Enriched	30380C
Company D-Classic 1	5820KJ
Company D-Classic 2	7200J
Company E (soymilk powder)	1100M
Company F-Light	5730KJ
Company F-Premium	3530L
Company G-Soy formula powder	3580L

* Soymilk with 1 or 2 designations were samples purchased six months apart. All soymilk except two powder as designated are liquid soymilk packaged either in milk cartons, Tetrapak®, or plastic bottles. Data are means of two replicates. Means followed by different letters are significantly different at $p < 0.05$.

Potential Health Benefits of Trypsin Inhibitors

Departing from the traditional view of trypsin as antinutrients, great attention has been paid in the exploration of trypsin inhibitors as health-promoting agents. A literature search using ACS SciFinder® database showed that more than one hundred articles have been published on the potential health benefits of protease inhibitors, including both Bowman-Birk and Kunitz types of the trypsin inhibitors in plant seeds. Most reported studies have been conducted using *in vitro*, cellular or animal models. However, human clinical studies on the cancer prevention of soy Bowman-Birk inhibitors have been an on-going research of Dr. A. R. Kennedy's group at the University of Pennsylvania, and results have not been published. Two recent reviews have been written to summarize the potential health benefits of Bowman-Birk inhibitor (*12*, *14*). However, the concentrations of the inhibitors used for health benefit studies are generally much lower than the large concentrations of the inhibitors naturally present in unheated soybeans or soymilk

(*38*). Therefore, the high level of trypsin inhibitor activities that are damaging to pancreas and digestion of proteins may not be relevant as compared to the low concentrations used in anticancer studies. From these points of view, the need to inactivate most inhibitor activities in soy foods is justified. A very recent report that both lunasin and Bowman-Birk inhibitor have complementary roles, in which lunasin is the actual substance that possesses a higher anticancer effect; however, the co-existing protease inhibitors function to protect the lunasin from being digested so that lunasin could be absorbed intact (*39*). However, current information on how much the protease inhibitors is needed for the protection of lunasin is not available. Once a desirable balanced level (range of concentrations) of Bowman-Birk inhibitor and Kunitz trypsin inhibitors are clearly known to have anticarcinogenic and/or other health properties in humans and have no adverse physiological effect against pancreas or protein digestibility, soymilk processing research must be directed to selectively eliminate the excessive soybean trypsin inhibitor to that balanced level and at that level the product is also of good flavor. Our research has contributed to the understanding of the behavior of TI, soy odor and protein extraction yield by heat processing, and would be of interest to the soy food industry for designing specific thermal processing technologies and products with the maximal food flavor quality, economic benefits and selective TI values for specific target health promotion, such as cancer prevention or others.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Acknowledgments

This project is a part of ND AES Hatch Project 1905. North Dakota Soybean Council and USDA CSREES-NRI CGP No. 2006-00907 provided funding for this project.

References

1. Food Labeling: Health Claim; Soy Protein and Coronary Heart Disease. FDA. 21 CFR Pt. 101. Final Rule. *Fed. Regist.* **1999**, *64* (206), 57700−57733.
2. Yuan, S. H.; Chang, S. K. C. Selected beany odors in soymilk as affected by chemical composition, and lipoxygenases in five soybean materials. *J. Agric. Food Chem.* **2007**, *55*, 426–427.
3. Yuan, S. H.; Chang, K. C. Selected odor compounds in soymilk as affected by soybean materials and direct steam injection. *J. Food Sci.* **2007**, *72*, S481–486.
4. Liener, I. E. Legume toxins in relation to protein digestibility: A review. *J. Food Sci.* **1976**, *41*, 1076–1081.
5. Liener, I. E.; Kakade, M. L. Protease Inhibitors. In *Toxic Constituents of Plant Foodstuffs*; Liener, I. E., Ed.; Academic Press: New York, 1980; pp 7−71.
6. Rackis, J. J.; Gumbamann, M. R. Protease Inhibitors: Physiological Properties and Nutritional Significance. In *Antinutrients and Natural*

Toxicants in Foods; Ory, R. L., Ed.; Food and Nutrition Press: Westport, CT, 1981; Chapter 12, pp 203−237.

7. DiPietro, C. M.; Liener, I. E. Heat inactivation of the Kunitz and Bowman-Birk soybean protease inhibitors. *J. Agric. Food Chem.* **1989**, *37*, 39–44.
8. Rouhana, A.; Adler-Nissen, J.; Cogan, U.; Frokiaer, H. Heat inactivation kinetics of trypsin inhibitors during high temperature-short time processing of soymilk. *J. Food Sci.* **1996**, *61*, 265–269.
9. Hackler, L. R.; Van Buran, J. P.; Streinkraus, K. H.; Rawi, I.; Hand, D. B. Effect of heat treatment on nutritive value of soymilk protein fed to weanling rats. *J. Food Sci.* **1965**, *30*, 723–38.
10. Hackler, L. R.; Stillings Amino acid composition of heat-processed soymilk and its correlation with nutritive value. *Cereal Chem.* **1967**, *44*, 70–77.
11. Luttrell, W. R.; Wei, L. S.; Nelson, A. I.; Steinberg, M. P. Cooked flavor in sterile Illinois soybean beverage. *J. Food Sci.* **1981**, *46*, 373–376, 382.
12. Clemente, A; Domoney, C. Therapeutic properties of legume protease inhibitors from the Bowman-Birk family. *Recent Prog. Med. Plants* **2008**, *20*, 397–417.
13. Kenndy, A. R.; Zhou, Z.; Donahue, J. J.; Ware, J. H. Protection against adverse biological effects induced by space radiation by the Bowman-Birk inhibitor and antioxidants. *Radiat. Res.* **2006**, *166*, 327–332.
14. Losso, J. N. The biochemical and functional food properties of the Bowman-Birk inhibitor. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 94–118.
15. Friedman, M.; Brandon, D. L. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **2001**, *49*, 1069–1086.
16. Reseland, J. E.; Holm, H.; Jacobsen, M. B.; Jenssen, T. G.; Hanssen Proteinase inhibitors induce selective stimulation of human trypsin and chymotrypsin secretion. *J. Nutr.* **1996**, *126*, 634–642.
17. Kwok, K. C.; Qin, W. H.; Tsang, J. C. Heat inactivation of trypsin inhibitors in soymilk at ultra high temperatures. *J. Food Sci.* **1993**, *58*, 859–862.
18. Kwok, K. C.; Liang, H. H.; Niranjan, K. Mathematical modeling of the heat inactivation of trypsin inhibitors in soymilk at 121-154C. *J. Sci. Food Agric.* **2002**, *82*, 243–247.
19. Kwok, K. C.; Liang, H. H.; Niranjan, K. Optimizing conditions for thermal processes of soymilk. *J. Agric. Food Chem.* **2002**, *50*, 4834–4838.
20. Guo, Q. C.; Liang, H. H.; Qin, W. H. The research on trypsin inhibitor activity for commercial soy beverage. *China Dairy Ind.* **1997**, *25* (6), 8–10.
21. Liener, I. E. Trypsin inhibitors: Concern for human nutrition or not? *J. Nutr.* **1986**, *116*, 920–923.
22. Johnson, L. A.; Deyoe, C. W.; Hoover, W. J.; Schwenke, J. R. Inactivation of trypsin inhibitors in aqueous soybean extracts by direct steam infusion. *Cereal Chem.* **1980**, *57*, 367–379.
23. Johnson, L. A.; Hoover, W. J.; Deyoe, C. W.; Erickson, L. E.; Johnson, W. H.; Schwenke, J. R. Modeling the kinetics of heat inactivation of trypsin inhibitors during steam-infusion cooking of soymilk. *Trans. ASAE* **1980**, *23*, 1326–1329.

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

Downloaded by DUKE UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch002

24. Yuan, S. H.; Chang, S. K. C. Elimination of trypsin inhibitor activity and beany flavor in soy milk by consecutive blanching and ultrahigh-temperature (UHT) processing. *J. Agric. Food Chem.* **2008**, *56*, 7957–7963.
25. Prabhakaran, M. P.; Perera, C. O. Effect of extraction methods and UHT treatment conditions on the level of isoflavones during soymilk manufacture. *Food Chem.* **2006**, *99*, 231–237.
26. Lo, W. Y. L.; Steinkraus, K. H.; Hand, D. B. Heat sterilization of bottled soymilk. *Food Technol.* **1968**, *22*, 787–789.
27. Kakade, M. L.; Rackis, J. J.; Mcgee, J. E.; Puski, G. Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. *Cereal Chem.* **1974**, *51*, 376–382.
28. Kakade, M. L.; Simons, N.; Liener, I. E. An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem.* **1969**, *46*, 518–526.
29. Anthon, G. E.; Barrett, D. M. Colorimetric method for the determination of lipoxygenase activity. *J. Agric. Food Chem.* **2001**, *49*, 32–372001.
30. *Official Methods of Analysis*, 17th ed.; AOAC International: Gaithersburg, MD, 2000.
31. *SAS/STAT 9.1 User's Guide*; SAS Institute, Inc.: Cary, NC, 2005.
32. Miyagi, M.; Shinjo, S.; Nishida, R.; Miyagi, C.; Takamatsu, K.; Yamamoto, T.; Yamamoto, C. Trypsin inhibitor activity in commercial soybean products in Japan. *J. Nutr. Sci. Vitaminol.* **1997**, *43*, 575–580.
33. Wallace, G. M.; Bannatyne, W. R.; Khaleque, A. Studies on processing and properties of soymilk. II. Effect of processing conditions on the trypsin inhibitor activity and digestibility *in vitro* of protein in various soymilk preparations. *J. Sci. Food Agric.* **1971**, *22*, 526–530.
34. Hoie, LH; Sjoholm, A; Guldstrand, M, F.; Zunft, HJ; Lueder, W; Graubaum, HJ; Gruenwald, J. Ultra heat treatment destroys cholesterol-lowering effect of soy protein. *Int. Food Sci. Nutr.* **2006**, *57*, 512–519.
35. Su, G; Chang, K. C. Trypsin inhibitor activity, *in vitro* digestibility, and sensory quality of meat-like yuba products as affected by processing. *J. Food Sci.* **2002**, *67*, 1260–1266.
36. Wilkens, W. F.; Mattick, L. R.; Hand, D. B. Effect of processing method on oxidative off-flavors of soybean milk. *Food Technol.* **1967**, *21*, 1630–1633.
37. Endo, E.; Ohno, M.; Tanji, K.; Shimada, S.; Kaneko, K. Effect of heat treatment on the lipid peroxide content and Aokusami (beany flavor) of soymilk. *Food Sci. Technol. Res.* **2004**, *10*, 328–333.
38. Liener, I. E. Possible adverse effects of soybean anticarcinogens. *J. Nutr.* **1995**, *125*, 744S–750S.
39. Hsieh, C. C.; Hernandez-Ledesma, B.; Jeong, H. J.; Park, J. H.; de Lumen, B. O. Complementary roles in cancer prevention: Protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS One* **2010**, *5* (1), e8890. www.plosone.org.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Chapter 3

Heating Sequence and Calcium Lactate Concentration Effects on *in Vitro* Protein Digestibility and Oil Release in Emulsion Stabilized by Preheated Soy Protein and Caseinate

Nantarat Na Nakornpanom,[1] Pranithi Hongsprabhas,[2] and Parichat Hongsprabhas*,[1]

[1]Department of Food Science and Technology, Kasetsart University, Bangkok 10900, Thailand
[2]Department of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
***parichat.h@ku.ac.th**

This study investigated the influences of heating sequence (one-stage vs. two-stage heating processes) and Ca lactate concentration (0-100 mM) on protein digestibility and oil released from reconstituted oil-in-water (o/w) emulsions under *in vitro* peptic and tryptic digestion. The high-calorie emulsions contained 1.0 kcal/mL. Caloric distributions from a carbohydrate:protein:lipid ratio of 55:15:30 were prepared by fabricating protein matrices with different characteristics at the oil-water interface and in the bulk aqueous phase, using heated mixed sodium caseinate (SCN) and soy protein isolate (SPI) at a ratio of 0.7:0.3. The preheating step prior to emulsification resulted in the adsorption of 7S globulins, phosphorylated caseins, and acidic 11S globulins at the oil-water interface. The reheating applied to the emulsion resulted in the disappearance of 7S globulins and the adsorption of protein aggregates with high molecular weight (>250 kDa) at the interface during peptic digestion. This did not occur in the emulsion prepared by the one-stage heating process. The two-stage heating process thus resulted in a slow release of peptides in the aqueous phase, and

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

a long lag phase for oil release during peptic digestion. The study indicated that the rate of nutrient release can be controlled by fabricating different protein matrix characteristics at the interface.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

The aging population, as well as consumers' concerns about health and well-being, has been the major driving forces for food manufacturers in recent years. Controlling the digestibility of macromolecules, such as proteins, lipids and starches, can influence postprandial metabolic behaviors: e.g. amino acid absorption, blood plasma triglyceride and glucose concentrations, etc. (*1*). The subsequent bioavailability of several nutrients is thus influenced by the fate of those microstructural elements assembled after they have been ingested (*1–4*).

Enteral formulae are liquid emulsions widely used in hospitals as complete nutrient products administrated into the gastrointestinal (GI) tract for patients who cannot swallow. They contain carbohydrates, proteins, lipids, vitamins and minerals that meet an individual's requirements when provided in adequate volume. The standard formula for an individual with normal GI function usually contains 1.0 kcal/mL and a caloric distribution from carbohydrate:protein:lipid of 37-55:15-25:30-45, if fluid intake does not need to be restricted. The calorie sources are usually in the form of biopolymers in the case of polymeric formulation. This is to control glycemic responses and decrease the osmolality of the formulae to lower the risk of osmotic diarrhea, which can occur if the osmolality is much higher than the isotonic osmolality of around 300 mOsmol/kg (*5*). Carbohydrates may be in the form of glucose syrup, maltodextrin, or other forms of complex carbohydrates. Protein sources in the commercial products include intact sodium caseinate (SCN) and/or soy protein isolate (SPI). The formulae also contain corn oil or soybean oil, in order to provide both an energy source and essential fatty acids.

Apart from the calorie-dense characteristics, the formulae need to be pathogen-free and have low viscosity (ca. 100-150 mPa·s) in order to flow through the 3.3-5.7 mm diameter tubing (10-16 French units; Fr) and enter the GI tract. The complete formulae usually contain mono-, di- and trivalent ions of high ionic strength (*6*). The presence of electrolytes to meet such requirements is a challenge for food manufacturers, who must optimize the process to avoid flocculation, coalescence, or even gelation during thermal processes and storage of the liquid emulsion.

Generally, emulsions stabilized by globular proteins are prone to flocculation and aggregation during heat treatment (*7*). An o/w emulsion stabilized by soy proteins shows a viscosity as high as 400 mPa·s when the protein concentration is high enough to be present in the aqueous phase (*8*). In addition, the protein quality and digestibility of SPI-containing enteral formulae are slightly lower than those of SCN-based ones (*9*). Although SCN has been extensively used as an excellent emulsifier in protein-stabilized emulsion, it is quite sensitive to ionic calcium (*10–14*). The phosphorylated forms of casein make the SCN-stabilized

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

emulsion prone to creaming and increased viscosity when ionic calcium is added above 10 mM (*12*, *13*).

The major proteins in SPI are 11S globulins and 7S globulins, which represent 34% and 27% of the proteins in SPI, respectively. 7S globulins are trimeric glycoprotein with a molecular weight (MW) between 140-170 kDa. It is composed of three types of subunit: namely α', α, and β. The MWs of these subunits are 58, 57 and 42 kDa, respectively. The α' and α subunits have isoelectric pH around 5.8-6.2. 7S globulins contain four sulfhydryl groups (SH) and two intramolecular disulfide bonds (SS). The subunits are linked mainly by ionic interactions and disulfide bonds (*15*). The denaturation temperature of 7S globulins is around 70 °C (*16–18*).

11S globulins are heterogeneous oligomer with MW 340-375 kDa. They consist of six subunits: i.e. acidic subunit (A) and basic subunit (B). The acidic subunits in 11S globulins have MW around 37-45 kDa, and their isoelectric pH ranges between 4.2-4.8. The MW of the basic subunits range between 18-20 kDa, and their isoelectric pH is quite high: 8.0-8.5. It has been determined that the 11S subunits are linked by disulfide bonds (*19*). The denaturation temperature of 11S globulins is around 90-96 °C (*16–18*).

Unlike the compact structure of soy globular proteins, caseins are much more flexible since they lack the ordered secondary and tertiary structures. Four individual caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) are major polypeptides in micelles, with MWs of 22, 25, 24 and 19 kDa, respectively (*20*). They are found in a proportion of 4:1:4:1 (*11*). Both α_{s1}- and α_{s2}-caseins contain 8-9 and 10-13 phosphoseryl residues/mole, respectively; while β-casein contains 5 phosphoseryl residues/mole, and κ-casein has only 1-2 phosphoseryl residues/mole. Only α_{s2}- and κ-casein contain cysteine; while α_{s1}- and β-casein lack both cysteine and cystine (*11*). In nature, these major caseins form the quaternary structure of casein micelles *via* Ca-phosphate linkages and hydrophobic interactions. However, the commonly used form of caseins on a commercial scale is SCN. It is soluble at a neutral pH. SCN is composed mainly of phosphorylated caseins, which have different physicochemical properties from those of micellar casein. The isoelectric pH of phosphorylated caseins in SCN ranges between 3.5 and 4.0, a much lower pH than that of individual casein (*21*).

This study hypothesized that heat treatment at a temperature higher than the denaturation temperature of 7S globulins but lower than that of 11S globulins could selectively alter most of the 7S subunits and change the physicochemical properties of the heated mixed SPI-SCN co-aggregates. This could result in the overall emulsifying characteristics of the mixed proteins, particularly the partitioning of polypeptides at the oil-water interface and in the bulk aqueous phase. Further alterations of the interface character and protein distribution may be introduced by additional heating. Such alterations of the proteins at the interface may influence the digestibility of proteins in the emulsion and the stability of the emulsion under *in vitro* digestion, which in turn affect the rates of protein and lipid digestion and absorption. Insights into the interplay between food formulation, processing, and subsequent microstructure of the o/w emulsion during *in vitro* digestion may help in understanding the behavior of the digest in the GI tract, as well as the rates of digestion and absorption of the nutrients.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Materials and Methods

Materials

Protein sources used were: commercial SPI (PROFAM 974, Archer Daniels Midland; Decatur IL, USA) containing 6.6% moisture, 82.1% protein (Nx6.25) and 0.5% fat; and commercial SCN (High Viscous, Gansu Hualing Milk Products Group; Gansu, China) containing 6.9% moisture, 80.6% protein (Nx6.25) and 0.4% fat (*22*). Food grade cassava starch (Jade Leaf, Bangkok Interfood Co.; Bangkok, Thailand) containing 11.2% moisture, 0.2% protein (Nx6.25) and 88.6% carbohydrate and cassava maltodextrin with dextrose equivalent of 10 (Neo-Maldex®, Neotech Food Co.; Bangkok, Thailand) was used as a carbohydrate source. Refined rice bran oil (King, Thai Edible Oil; Bangkok, Thailand) was used as the sole lipid source.

Preparation of O/W Emulsion Powder

A liquid emulsion providing 1.0 kcal/mL was prepared. It was composed of 3.8% (w/v) protein from SCN and SPI at a ratio of 0.7:0.3; cassava starch 2.0% (w/v); and cassava maltodextrin 11.8% (w/v) dispersed in 3.34 L of water at pH 3.0 (adjusted by 1 M lactic acid). The suspension was preheated at 80 °C for 30 min, cooled to room temperature (27 °C), and emulsified with rice bran oil to provide 3.3% lipid content using a high-speed colloid mill (2F-colloid mill, APV Gaulin, Inc.; Wilmington MA, USA) for 3 min at 24000 rpm. Ca lactate powder was added to the emulsion at 38° to 40 °C to obtain the final concentrations of Ca lactate of 0, 25 and 100 mM. This method of liquid emulsion preparation was designated as the “one-stage heating process.”

In the “two-stage heating process” the emulsion was further re-heated at 80 °C for 30 min to obtain a salt-induced protein aggregate structure at the interface and in bulk aqueous phase (*23*) prior to drying. The emulsions prepared by the one-stage and two-stage heating processes were spray-dried in a spray dryer (GEA Niro, Niro A/S; Soeborg, Denmark). The liquid was fed at 16 mL/min and dried using inlet air of 160 °C and outlet air of 85 °C, with the flow of drying air of 1 m^3/min. The dry powder was stored at -20 °C prior to analysis.

In Vitro Protein Digestion of the Reconstituted Liquid O/W Emulsion

In vitro protein digestion was evaluated using the method described by Glahn et al. (*24*). The reconstituted liquid emulsion was prepared in a 250 mL beaker by dispersing 20.8 g powder in 100 mL distilled water at 75 °C, to provide an emulsion with 1.0 kcal/mL; the emulsion was then cooled. The pH was adjusted to 2.0 using 5 M HCl, and hydrolyzed by pepsin (EC 3.4.23.1, Sigma Chemical; St. Louis MO, USA) at 37 °C using an enzyme to substrate ratio of 1:17. Peptic digestion was allowed to proceed for 150 min. Samples were withdrawn at specified time intervals, adjusted to pH 7.0 by 2 M NaOH, and further hydrolyzed with trypsin (EC 3.4.21.4, Sigma Chemical) using an enzyme to substrate ratio of 1:100 at 37 °C. Bile extract (Sigma-Aldrich, St. Louis MO, USA) in a 0.1 M phosphate buffer (pH 7.0) was added to obtain a final concentration of bile acid in the emulsion of 0.7 μmol/mL.

Emulsion Stability

The method for evaluating emulsion stability was carried out using a centrifugal method (*25*). Before digestion by pepsin and trypsin, Oil Red "O" (Fluka Chemika; St. Louis MO, USA) was added to the emulsion to indicate the oil phase. The hydrolyzed emulsion was centrifuged at 12000 rpm for 10 min by a microhematocrit centrifuge (KHT-400, Gemmy Industrial Corp.; Taipei, Taiwan). After centrifugation, the hydrolyzed emulsion was separated into four layers: a red oil layer on the top, an opaque cream layer, a translucent aqueous layer, and opaque sediment. The separated oil height and total height were measured with a vernier caliper, and the results were reported as oil phase height (%) compared with total height.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Protein at the Oil-Water Interface and in the Bulk Aqueous Phase

Samples (1.0 mL) of hydrolyzed emulsion, or digest, were pipetted into Eppendorf tubes and centrifuged at 14000 rpm for 10 min by a microcentrifuge (Labnet Spectrafuge 16M, Labnet International Inc.; Woodbridge NJ, USA) to separate the cream phase (top) from the aqueous phase (bottom). The cream was separated and dried on filter paper (Whatman No. 1, Whatman International Ltd.; Maidstone, UK) and then resuspended in 0.5 mL of extraction buffer containing 0.5 M Tris-HCL (pH 6.8), 10% glycerol and 0.1% (w/v) SDS (*26*). The concentrations of extracted proteins from the cream phase and the aqueous phase were determined by Lowry's method (*27*).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The MW profiles of proteins in the re-suspended cream phase and the aqueous phase were carried out using SDS-PAGE (*28*) in 4% stacking gel and 15% separating gel. The continuous buffer contained 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS for separating gel; and 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS for stacking gel. The running buffer contained 0.024 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.3. Aliquots of the aqueous were added to dissociating buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, with or without 5% β-mercaptoethanol, 1% (w/v) bromophenol blue). Aliquots of the re-suspended cream were added to 20 μL of 1% (w/v) bromophenol blue. Each solution was heated at 100 °C for 3 min, cooled, and centrifuged at 5000 rpm (Labnet Spectrafuge 16M, Labnet International) for 5 min to remove insoluble material. An aliquot of the sample solution containing 0.03 mg protein and 4 μL wide range MW standards was loaded into each well. Electrophoresis was run at a constant voltage of 150 V for the cream phase and at a constant current of 25 mA for the aqueous phase. Gel slabs were fixed and stained simultaneously using Bio-Rad Coomassie blue R-250 stain solution (40% methanol, 10% acetic acid, 0.1% Coomassie blue R-250) for 30 min, and then de-stained by Bio-Rad Coomassie blue R-250 de-staining solution for 5 h with 2-3 changes of de-staining solution. The MW of proteins was determined using full-range rainbow MW markers of ~10 to 250 kDa (RPN 8000, Amersham Biosciences UK Ltd.; Buckinghamshire, UK) as the MW standards.

Determination of Peptide Released during Digestion

Samples (1.0 mL) of the digest were pipetted into Eppendorf tubes. The aqueous phase was collected by centrifugation at 14000 rpm for 10 min in a microcentrifuge (Labnet Spectrafuge 16M, Labnet International), and removed with a syringe. The peptides released in the aqueous phase were obtained by mixing the aqueous phase with trichloroacetic acid (TCA) at a final concentration of 10% (w/v) TCA. The solutions were allowed to stand for 10 min at room temperature. Insoluble protein was removed by centrifugation at 14000 rpm for 10 min. Supernatants were collected and analyzed for protein content (*N*x6.25) by the Kjeldahl method (*22*).

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Microstructure

The microstructure of the emulsions taken during digestion was observed using confocal laser scanning microscopy (CLSM) (LSM 5 PASCAL, Carl Zeiss Pte. Ltd.; Jena, Thüringen, Germany). Rhodamine B (0.01% in 95% ethanol) was added to the emulsions or digest. After incubation for 5 min, samples were loaded onto well slides and observed for the location of fluorescent-labeled proteins. A He/Ne laser was used as a laser source, at an excitation wavelength of 543 nm. Micrographs were acquired at 1024x1024 pixels using the LSM 5 PASCAL program.

Statistical Analysis

The high-caloric liquid emulsions and their spray-dried products were prepared in two separate trials. Each trial was run and evaluated in duplicates. The data were analyzed by analysis of variance (ANOVA) with significance at $p<0.05$. Significant difference among mean values was determined by Duncan's multiple range test. All statistical analyses were performed using the SPSS Software Version 12.

Results and Discussion

Effect of Heating Schemes on *in Vitro* Digestion

Reconstituted emulsions containing 25 mM of Ca lactate, which were prepared by different heating schemes, were prone to differences in peptic digestion: in terms of the protein content deposited at the oil-water interface in the cream phase, and in the lag phase before oil was released from the emulsion during peptic digestion (Figure 1). Both heating schemes resulted in a similar content of TCA-soluble protein in the aqueous phase. The protein in the aqueous phase increased from 0.09% to 1.18% during the first 15 min of digestion, and then plateaued at that concentration (Figure 1a). Nevertheless, the protein contents in the cream phase were different when the emulsions were prepared using different heating schemes. The cream phase of the emulsion prepared by the one-stage heating process had a protein content within a range of 0.52%-0.70% during the first 60 min of digestion by pepsin at pH 2.0 (Figure 1b). However, the protein content in the cream phase of the emulsion prepared by the two-stage heating process was raised from 0.30% to 1.06% during the first 60 min of digestion ($p<0.05$). This increase in protein content in the cream phase was likely to be responsible for the delayed release of oil during peptic digestion from the emulsion prepared by the two-stage heating process (Figure 1c).

Figure 2 illustrates the CLSM of emulsions prepared by different heating schemes, before and after peptic digestion. The reconstituted emulsion prepared by the one-stage heating process (Figure 2a) had a much larger oil droplet size (i.e., mostly 20 μm) than the ones from the two-stage heating process, in which the oil droplets were mostly less than 5 μm (Figure 2e). Upon acidification of the reconstituted emulsion to pH of 2.0 prior to digestion, the oil droplets flocculated due to the aggregation of protein at the interface. The microstructure of the protein at the interface of emulsions prepared by one-stage and two-stage heating processes showed different manners of aggregation. In the emulsion prepared by the one-stage heating process, oil droplets with a thin coat of protein flocculated heavily when the pH was adjusted to 2.0, and the globule shape of the droplet was retained (Figure 2b). However, proteins in the emulsion prepared by the two-stage heating process aggregated greatly and entrapped the oil droplets within the matrix (Figure 2f). After being digested by pepsin for 15 min, various sizes of oil droplets with a thick interfacial protein layer were observed in the digest prepared by the one-stage heating process (Figure 2c). This phenomenon was not evident in the digest prepared by the two-stage heating process. Here, the protein matrix disintegrated, revealing individual small oil droplets with thin interfacial proteins (Figure 2g). In the emulsion prepared by the one-stage heating process, after 60 min of digestion, both the microstructure of the interfacial protein film in the digest and the oil droplet size remained unchanged (Figure 2d). However, the oil droplets in the digest prepared by the two-stage heating process were larger, and were bridged by the protein matrix, forming clumps (Figure 2h).

The different heating schemes employed during the preparation of emulsions resulted in preferential adsorption of proteins at the oil-water interface (Figure 3). The majority of the proteins at the interface or in the cream phase had a MW between 25-30 kDa (Figures 3a, 3b; lane 1) before digestion. This included both phosphorylated caseins and acidic subunits of 11S globulins, which were found in emulsions prepared by both heating schemes. However, the cream phase of the emulsion prepared by the one-stage heating process contained more proteins with a MW above 50 kDa than did the emulsion prepared by the two-stage heating process (Figure 3b; lane 1). These were likely to be 7S globulins and co-aggregates resulting from the preheating treatment of the protein mixture prior to emulsification.

Further digestion by pepsin hydrolyzed most of the polypeptides to a MW of less than 35 kDa during the first 60 min of digestion. But the second heating scheme employed in the two-stage heating process generated high MW aggregates

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

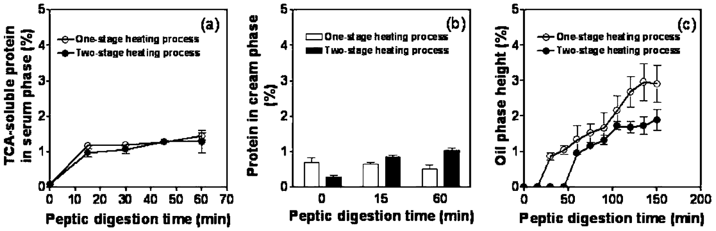


Figure 1. Effect of heating schemes on (a) soluble protein content in aqueous phase; (b) protein content in cream phase; and (c) oil released from the digest. The emulsions investigated contained 25 mM Ca lactate. Bars represent standard deviation.

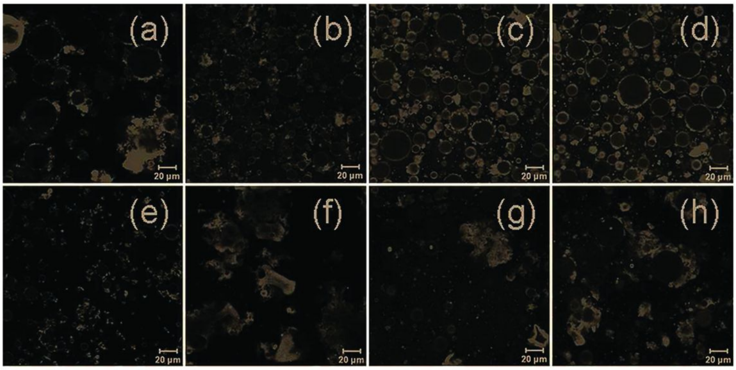
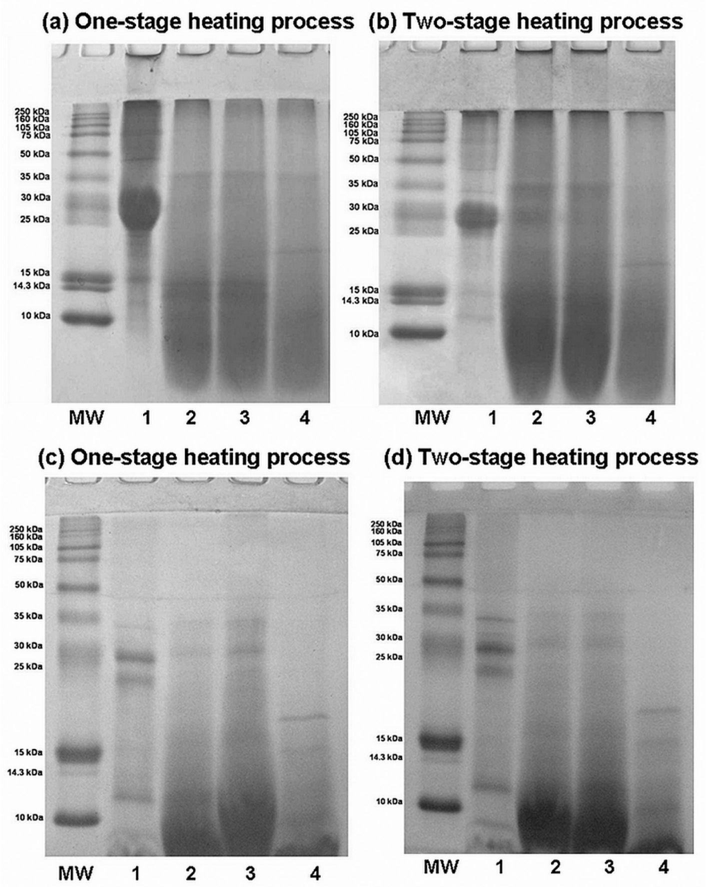


Figure 2. CLSM of o/w emulsions containing 25 mM Ca lactate prepared by (a-d) one-stage heating process and (e-f) two-stage heating process. (a, e) reconstituted emulsions; (b, f) reconstituted emulsions adjusted to pH 2.0; (c, g) 15 min peptic digestion; and (d, h) 60 min peptic digestion. Protein was fluoresced in light color by rhodamine B.

(more than 250 kDa) that precipitated in the wells (Figure 3b; lanes 2 and 3). This fraction was still present even after digestion by pepsin for 60 min, but disappeared after tryptic digestion (Figure 3b; lane 4). Such large MW precipitates were not present in the digest prepared by the one-stage heating process (Figure 3a; lanes 2 and 3).

Altered heating schemes resulted in a slight difference in the polypeptides in the aqueous phase (Figures 3c, 3d) compared to those in the cream phase (Figures 3a, 3b). Polypeptides with MW slightly below 25 kDa and between 10 and 14.3 kDa were clearly observed in the aqueous phase (Figures 3c, 3d; lane 1), suggesting the presence of phosphorylated caseins and basic subunits of 11S globulins. However, only the emulsion prepared by the two-stage heating process showed the presence of acidic subunits of 11S globulins in the aqueous phase. Over time, the polypeptides in both aqueous and cream phases were hydrolyzed by trypsin into peptides with MW of less than 15 kDa.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003


(a) One-stage heating process (b) Two-stage heating process

(c) One-stage heating process (d) Two-stage heating process

Figure 3. Effect of heating schemes on protein MW profiles. (a, b) protein cream phase, and (c, d) protein in aqueous phase. Lane 1 = 0 min peptic digestion; lane 2 = 15 min peptic digestion; lane 3 = 60 min peptic digestion; and lane 4 = 15 min peptic digestion followed by 30 min tryptic digestion.

Although oil was not released after digestion by pepsin for 15 min, the digest released oil after being further digested by trypsin for another 30 min, both in the absence and presence of bile acid (Table 1). The heating scheme did not significantly alter the amount of oil released after tryptic digestion (p>0.05). It is likely that the rate-limiting step for oil release is regulated by peptic digestion in the stomach. The released free oil can then be emulsified by bile acid and digested in the upper intestine.

Effect of Calcium Concentration on *in Vitro* Digestibility

Concentrations of Ca lactate within a range of 0 to 100 mM could also be used to alter the protein content in the cream phase of the reconstituted emulsion (Figure 4). In the absence of Ca lactate, the protein content in the cream phase remained unchanged during 60 min of peptic digestion (pH 2.0) (Figure 4a). However, the

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

addition of Ca lactate resulted in an increase in protein content in the cream phase during the first 60 min of peptic digestion. The cream phase of the digest had the highest protein content in the presence of 25 mM Ca lactate. However, over a peptic digestion time of 150 min, the protein content in the cream phase of digest containing added Ca lactate, as well as the oil release characteristics of all digest during peptic digestion, remained unchanged (Figure 4b).

The reconstituted emulsion prepared in the absence of Ca lactate (Figure 5) had larger oil droplet sizes (Figure 5a) and more proteins dispersed in the bulk aqueous phase than did the emulsions containing 25 and 100 mM calcium lactate (Figures 5e, 5i). Upon acidification to pH 2.0, the proteins clumped into large aggregates, and flocculation of the oil droplets was observed – particularly in the emulsion containing 25 mM of Ca lactate (Figure 5f). Disintegration of the flocculated protein matrix and the oil droplets, as well as an increase in oil droplet size, became evident after the emulsion was digested for 15 min by pepsin (Figures 5c, 5g, 5k). After 60 min of peptic digestion, the protein fraction in the digest having 100 mM Ca lactate re-aggregated into large particles with larger clumps than the others (Figure 5l).

Table 1. Effect of heating process and bile acid on oil released from digest containing 25 mM Ca lactate

Digestion scheme	*Oil phase height (%)*[a]	
	One-stage heating process	*Two-stage heating process*
15 min pepsin	None	None
15 min pepsin + 30 min trypsin	1.86^{abc}	1.58^{c}
15 min pepsin + 30 min trypsin + bile acid	2.16^{ab}	1.92^{abc}
15 min pepsin + 60 min trypsin	2.09^{ab}	1.73^{bc}
15 min pepsin + 60 min trypsin + bile acid	2.28^{a}	1.85^{abc}

[a] Means followed by different superscripts are significantly different (p<0.05).

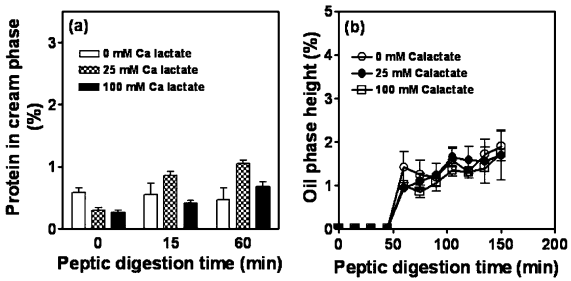


Figure 4. Effect of Ca lactate concentration on (a) protein content in cream phase and (b) oil released from the digest. The emulsions were prepared by the two-stage heating process. Bars represent standard deviation.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

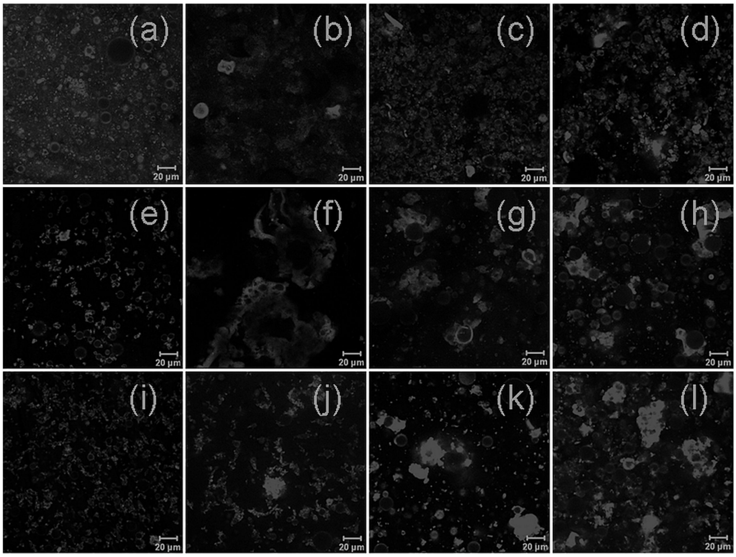


Figure 5. CLSM of o/w emulsions prepared by the two-stage heating process and containing (a-d) 0 mM; (e-f) 25 mM; and (i-l) 100 mM Ca lactate. (a, e, i) reconstituted emulsions; (b, f, j) reconstituted emulsions adjusted to pH 2.0; (c, g, k) 15 min peptic digestion; and (d, h, l) 60 min peptic digestion. Protein was fluoresced in red by rhodamine B.

Table 2. Effect of Ca lactate concentration and bile acid on oil released from digest prepared by the two-stage heating process

Digestion scheme	*Oil phase height (%) at different Ca lactate concentration*[a]		
	0 mM	*25 mM*	*100 mM*
15 min pepsin	None	None	None
15 min pepsin + 30 min trypsin	1.01[b]	1.58[a]	0.97[b]
15 min pepsin + 30 min trypsin + bile acid	1.16[b]	1.92[a]	None
15 min pepsin + 60 min trypsin	1.11[b]	1.73[a]	1.02[b]
15 min pepsin + 60 min trypsin + bile acid	1.14[b]	1.85[a]	1.03[b]

[a] Means followed by different superscripts are significantly different ($p<0.05$).

The presence of 25 mM Ca lactate resulted in the highest oil phase release (Table 2). After trypsin digestion for 30 min, the digest containing 100 mM of Ca lactate was able to withstand both the presence of bile acid and protein digestion. No detectable oil separation was observed in this case. It is possible that the strong protein matrix at the interface entrapped the oil droplet and protected it from being emulsified by bile acid.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Preferential adsorption of soy and phosphorylated proteins at the interface were also observed as the Ca lactate concentration increased (Figure 6). In the cream phase of the emulsion with 100 mM Ca lactate added, there was an absence of polypeptides with MW above 50 kDa and between 25-30 kDa (mainly soy proteins and phosphorylated caseins, respectively) (Figure 6c). Polypeptides with a much lower MW, i.e. less than 15 kDa, were present in the cream phase of this emulsion. These polypeptides were not adsorbed at the oil-water interface of the emulsions prepared in the absence of Ca lactate and the one containing 25 mM Ca lactate (Figures 6a, 6b, respectively). Upon peptic and tryptic digestion, protein bands present in the cream phase of digest containing 0 mM of Ca lactate had very low intensity (Figure 6a; lanes 2, 3, 4) compared to those in the cream phase of digest containing 25 and 100 mM of calcium lactate. Further tryptic digestion of the latter hydrolyzed most of the polypeptides at the interface to a MW of less than 15 kDa.

Ca lactate concentration also resulted in a different polypeptide distribution in the aqueous phase (Figures 6d, 6e, 6f) compared to those in cream phase (Figures 6a, 6b, 6c). Polypeptides with a MW above 30 kDa were observed only in the aqueous phase of the emulsion prepared in the absence of Ca lactate. Polypeptides with MW slightly below 25 kDa and between 10 and 14.3 kDa were observed in the aqueous phase of emulsions containing 0 and 25 mM Ca lactate; they were also found, but with less intensity, in the aqueous phase of the emulsion containing 100 mM Ca lactate. The increase of Ca lactate to 100 mM may reduce the solubility of the phosphorylated caseins. It is likely that most of these precipitated in the sediment and did not participate in emulsion stabilization.

This study showed that phosphorylated caseins, acidic subunits of 11S globulins, and 7S globulins were preferentially adsorbed when the reconstituted emulsion was prepared by the one-stage heating process. The first heating step or preheating (80 °C for 30 min) of protein-carbohydrate suspensions prior to emulsification altered the mixed protein characteristics by selectively denaturing the 7S globulins, which have a denaturation temperature of around 70 °C (*15–17*). At 80 °C most of the 7S globulins were unfolded. Thus the 7S globulins could be incorporated with the phosphorylated caseins at the oil-water interface to a greater extent than could the folded 11S globulins.

In the reconstituted emulsion prepared by the two-stage heating process, however, the bands with less intensity of 7S globulins were present at the oil-water interface and not in the aqueous phase. The re-heating step employed in the two-stage heating process was likely to induce the 7S globulins, together with other proteins, to polymerize into large MW aggregates that did not enter the separating gel. It is possible that the large MW aggregates occurred due to the aggregation of the adsorbed protein at acidic pH, and/or due to the deposit of soluble proteins and peptides in the aqueous phase with the adsorbed protein at the interface. Thereby, the protein content at the interface of the digest increased during digestion.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

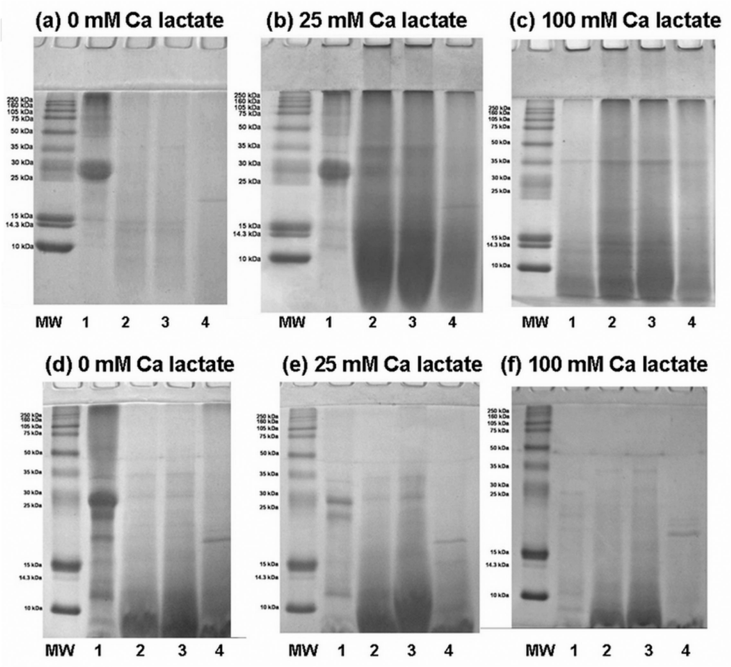


Figure 6. Effect of Ca lactate concentration on protein MW profiles. (a, b) protein in cream phase and (c, d) protein in aqueous phase. Lane 1 = 0 min peptic digestion; lane 2 = 15 min peptic digestion; lane 3 = 60 min peptic digestion; and lane 4 = 15 min peptic digestion followed by 30 min tryptic digestion.

Phosphorylated caseins were the main constituents of the protein matrix at the oil-water interface. These phosphorylated caseins were prone to further alteration by acidification to pH 2.0. This resulted in the flocculation of the oil droplets entrapped in the protein matrix, as observed under CLSM. After being digested by pepsin for 60 min, some of the oil droplets prepared by the two-stage heating process were bridged together. This was not observed in the digest prepared by the one-stage heating process. It is possible that different protein and peptide species at the interfaces of digest prepared by different heating schemes possessed different aggregation mechanisms.

This study suggests that the interfacial protein characteristics which slightly retard peptic digestion and oil release under stomach conditions could effectively regulate the entire *in vitro* digestion scheme. This could be done by using a two-stage heating process in the presence of 25 mM Ca lactate. Increasing the Ca lactate concentration could further alter the protein and peptide distribution at the interface and in sediment, due to the sensitivity of phosphorylated caseins to ionic Ca. This dynamic interplay of ingredients is crucial to the outcome of digestion, in terms of substrate assimilation and digestion rates of nutrients, which in turn may influence the entire process of digestion and absorption in the GI tract.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Conclusions

This study demonstrated that the heating sequence could be used to regulate the preferential adsorption of phosphorylated caseins and soy proteins at the oil-water interface during the preparation of high-caloric polymeric emulsions. Consequently, the proteins at the interface *in priori*, as well as the resulting proteins in the bulk aqueous phase, influenced the nature of protein aggregation; the coalescence of oil droplets during acidification; the deposit of proteins and peptides during *in vitro* protein hydrolysis; the lag phase before oil release; and the amount of released oil. This study also introduced a so-called "two-stage heating process" in the preparation of high-calorie emulsions. This can be used in the preparation of basic nutrient supplements in powdered form for further fortification of the mineral, vitamin and dietary fiber supplements used in hospitals to meet patients' requirements.

Acknowledgments

The authors gratefully acknowledge financial support from the National Center for Genetic Engineering and Biotechnology, Thailand, under Project no. BT-B-01-FG-11-4802; and from Kasetsart University Graduate School. Na Nakornpanom is grateful for a Ph.D. scholarship from the Thai Ministry of Education.

References

1. Lundin, L.; Golding, M.; Wooster, T. J. Understanding food structure and function in developing food for appetite control. *Nutr. Diet.* **2008**, *65* (Supplement 3), S79–S85.
2. Parada, J.; Aguilera, J. M. Food microstructure affects the bioavailability of several nutrients. *J. Food Sci.* **2007**, *72*, R21–R32.
3. Lentle, R. G.; Janssen, P. W. M. Physical characteristics of digesta and their influence on flow and mixing in mammalian intestine: A review. *J. Comp. Physiol., B* **2008**, *178*, 673–690.
4. McClements, D. J.; Decker, E. A.; Park, Y.; Weiss, J. Designing food structure to control stability, digestion, release and absorption of lipophilic food components. *Food Biophys.* **2008**, *3*, 219–228.
5. Pfeiffer, A.; Schmidt, T.; Kaess, H. The role of osmolality in the absorption of a nutrient solution. *Aliment. Pharmacol. Ther.* **1998**, *12*, 281–286.
6. Nanovic, L. Electrolytes and fluid management in hemodialysis and peritoneal dialysis. *Nutr. Clin. Pract.* **2005**, *20*, 192–201.
7. McClements, D. J. Protein-stabilized emulsions. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 305–313.
8. Roesch, R. R.; Corredig, M. Characterization of oil-in-water emulsions prepared with commercial soy protein concentrate. *J. Food Sci.* **2002**, *67*, 2837–2842.
9. Castillo, G.; Ángeles Sanz, M.; Ángeles Serrano, M.; Hernánsez, A. Influence of protein source, type, and concentration, and product form, on

the protein quality of commercial enteral formulas. *J. Food Sci.* **2002**, *67*, 328–334.

10. Agboola, S. O.; Dalgleish, D. G. Calcium-induced destabilization of oil-in-water emulsions stabilized by caseinate or by β-lactoglobulin. *J. Food Sci.* **1995**, *60*, 399–404.
11. Dalgleish, D. G. Structure-Function Relationships of Caseins. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds.; Marcel Dekker: New York, 1997; pp 199−223.
12. Dickinson, E.; Golding, M. Influence of calcium ions on creaming and rheology of emulsions containing sodium caseinate. *Colloids Surf., A* **1998**, *144*, 167–177.
13. Dickinson, E.; Davies, E. Influence of ionic calcium on stability of sodium caseinate emulsions. *Colloids Surf., B* **1999**, *12*, 203–212.
14. Lee, S. Y.; Morr, C. V.; Ha, E. Y. W. Structural and functional properties of caseinate and whey protein isolate as affected by temperature and pH. *J. Food Eng.* **1992**, *79*, 706–717.
15. Petrucelli, S.; Añón, M. C. Soy protein isolate components and their interactions. *J. Agric. Food Chem.* **1995**, *43*, 1762–1767.
16. Bainy, E. M.; Tosh, S. M.; Corredig, M.; Woodrow, L.; Poysa, V. Protein subunit composition effects on the thermal denaturation at different stages during the soy protein isolate processing and gelation profiles of soy protein isolates. *J. Am. Oil Chem. Soc.* **2008**, *85*, 581–590.
17. Sorgentini, D. A.; Wagner, J. R.; Añón, M. C. Effects of thermal treatment of soy protein isolate on the characteristics and structure-function relationship of soluble and insoluble fractions. *J. Agric. Food Chem.* **1995**, *43*, 2471–2479.
18. Zhang, H.; Takenaka, M.; Isobe, S. DSC and electrophoretic studies on soymilk protein denaturation. *J. Therm. Anal. Calorim.* **2004**, *75*, 719–726.
19. Utsumi, S.; Matsumura, Y.; Mori, T. Heterogeneity of soy bean glycinin. *Phytochemistry* **1981**, *20*, 585–589.
20. Farrell, H. M., Jr.; Jimenez-Flores, R.; Bleck, G. T.; Brown, E. M.; Butler, J. E.; Creamer, L. K.; Hicks, C. L.; Hollar, C. M.; Ng-Kwai-Hang, K. F.; Swaisgood, H. E. Nomenclature of the proteins of cow's milk: Sixth revision. *J. Dairy Sci.* **2004**, *87*, 1641–1674.
21. Jahaniaval, F.; Kakuda, Y.; Abraham, V; Marcone, M. F. Soluble protein fractions from pH and heat treated sodium caseinate: Physicochemical and functional properties. *Food Res. Int.* **2000**, *33*, 637–647.
22. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Gaithersburg, MD, 2000.
23. Hongsprabhas, P.; Barbut, S. Ca^{2+}-induced cold gelation of whey protein isolate: Effect of two-stage gelation. *Food Res. Int.* **1998**, *30*, 523–527.
24. Glahn, R. P.; Lai, C.; Hsu, J.; Thompson, J. F; Guo, M. R.; Van-Campen, D. R. Decreased citrate improves iron availability from infant formula: Application of an *in vitro* digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 256–264.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

25. McDermott, R. L.; Harper, W. J.; Whitley, R. A centrifugal method for characterization of salad dressing emulsions. *Food Technol.* **1981**, *35*, 81–82, 84–85, 87.
26. Manion, B.; Corredig, M. Interactions between whey protein isolate and soy protein fractions at oil-water interfaces: Effects of heat and concentration of protein in the aqueous phase. *J. Food Sci.* **2006**, *71*, E343–E349.
27. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
28. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **1970**, *227*, 680–686.

Downloaded by UNIV OF GUELPH LIBRARY on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch003

Chapter 4

Effects of Heating Temperature and Cooling Rate on Denaturation of Soymilk Protein

Makoto Shimoyamada,*,[1] Kimiko Tsuzuki,[2] Hiroaki Asao,[2] and Ryo Yamauchi[3]

[1]School of Food, Agricultural and Environmental Sciences,
Miyagi University, 2-2-1 Hatatate, Taihaku, Sendai, Miyagi 982-0215, Japan
[2]Marusan-ai Co., Ltd., 1 Arashita, Niki-cho, Okazaki, Aichi 444-2193, Japan
[3]Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
***shimoyam@myu.ac.jp**

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

In order to evaluate the effects of heating and cooling on soymilk protein, raw soymilk, which was squeezed before heating, was heated to 80, 100 or 115°C and then cooled at room temperature or -5°C. The surface SH content of the soymilk proteins decreased with increasing heating temperatures. Furthermore, the SH content of the soymilk that was cooled rapidly at -5°C was higher than that of the soymilk that was cooled slowly at room temperature. The surface hydrophobicity of the soymilk proteins was increased by heating, and the hydrophobicity was slightly higher as a result of cooling at -5°C compared with cooling at room temperature. The raw soymilk was first heated at 115°C (first heating) and then 80°C (second heating). After cooling in an ice bath, decreases in both the SH content and the hydrophobicity of the resulting soymilk were observed as a result of the second heating. This denaturation behavior is different from that of a protein solution prepared from defatted soybeans.

Soymilk is composed of protein suspensions, emulsions consisting of lipids and proteins, and others substances. Soymilk has beneficial qualities that improve human health, for example, lowering of serum lipids and cholesterol (*1*, *2*),

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

estrogen-like activity (*3*) and antioxidative activity (*4*). In Japan, about 200,000 tons of soymilk is consumed each year.

Soymilk is prepared from imbibed soybeans by milling, removing insoluble residue and heating. In the case of commercial soymilk, two or more heating steps are included to denature soymilk proteins for easy digestion, to inactivate lipoxygenase and other bioactive components, and to sterilize the soymilk for aseptic filling. It is thought that heating affects the structures and functional properties of proteins. It has been reported that proteins in soymilk form aggregates upon heating and interact differently with lipids (*5–7*), which affects the physical properties of tofu (*8*).

Regarding the heating and successive cooling and/or freezing of soymilk, Shimoyamada et al. (*9*) have reported that frozen storage allowed the soymilk to form a gel-like coagulate (freeze-gel). In this process, pre-cooling at -5 °C, which was carried out successively after heat treatment, was essential for the freeze-gelation of soymilk. Pre-cooling has been divided into two classifications, namely, supercooling and rapid cooling. Small globular ice crystals, which are formed by freezing supercooled soymilk, are effective at maintaining the gel-like structure of frozen and thawed soymilk (*10*). Through heating, the surface SH content and the surface hydrophobicity of proteins in the soymilk are altered. Rapid cooling increases the SH content and hydrophobicity of the heated soymilk. These results are attributed to the maintained reactivity of the heat-denatured protein molecules (*11*). These results imply that protein denaturation behavior is affected by cooling as well as heating.

In this study, the denaturation behavior of soymilk proteins was monitored after heating and cooling at various temperatures to estimate the effects of cooling rate on the thermal denaturation of soymilk proteins. Two-step heating at first a higher temperature and then a lower temperature can be considered a kind of slow cooling; therefore, the denaturation behavior of the soymilk sample was also monitored after two-step heating at two separate temperatures.

In order to estimate the effects of heating and cooling, three heating and two cooling temperatures were selected. At a heating temperature of 80°C, only β-conglycinin is denatured, and glycinin remains native. At 100°C, both β-conglycinin and glycinin are denatured as determined by DSC measurements (*12*, *13*). A temperature of 115°C was selected to consider the sterilization temperature of soymilk. Since a pressure cooker was used, the temperature was limited to 115 °C. Cooling at room temperature is referred to as slow cooling, and cooling at -5°C in a refrigerator is referred to as rapid cooling. In the latter case, the soymilk never froze due to supercooling.

The denaturation behavior and precipitation of soymilk proteins were measured after cooling the heated soymilk samples, and the effects of heating temperature and cooling rate were evaluated.

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

Materials and Methods

Preparation of Soymilk and Heat Treatment

Soybeans were soaked in water overnight at 4°C, and milled with water in a blender (6,000 rpm × 5 min, Type R, Teraoka Co., Osaka, Japan). The total volume of imbibed and milling water that was added to 30 g of the dry soybean was 300 mL. The milled soybeans were filtered with 5-ply gauze to afford raw soymilk at less than 60°C. The raw soymilk samples were heated at 80 or 100°C in a water bath, or at 115°C in a pressure cooker. The heated soymilk samples were cooled at -5°C in a refrigerator (rapid cooling) or at room temperature (slow cooling) for 2 h and then put in an ice-water bath (0°C) until the analyses were conducted.

Estimation of Precipitate from Heated Soymilk

After successive heating and cooling, the soymilk was refrigerated at 4°C for 10 days in a screw-capped test tube and then centrifuged at 1,500 × *g* for 30 min, and the supernatant was removed by placing the test tubes upside down for half a minute. The weight of the precipitate was used to calculate precipitation rate.

Protein Surface SH Content and Surface Hydrophobicity

The protein surface SH content was estimated by using 2,2′-dithiobis-(5-nitropyridine) (DTNP) (*14*, *15*). The samples were diluted to a volume of 2 mL with 0.1 mol/L phosphate buffer (pH 7.6) and then mixed with 0.5 mL of 5.0×10^{-4} mol/L DTNP ethanol solution. The samples were incubated at 25°C for 20 min, and 2.5 mL of 10% perchloric acid solution was added. The samples were centrifuged at 1,500 × *g* for 10 min to remove the protein. The resulting supernatant was passed through a 0.45 µm membrane filter, and the filtrate was analyzed by a spectrophotometer (NovaspecII, Pharmacia LKB Biotechnology, Uppsala, Sweden) at 386 nm.

The surface hydrophobicity of the soymilk proteins was measured using 8-anilino-1-naphtalene sulfonic acid (ANS) (*16*). The samples were diluted to a volume of 100 µl with 0.01 mol/L phosphate buffer (pH 7.0) and mixed with 20 µl of 8×10^{-3} M ANS solution and 4 mL of the pH 7.0 phosphate buffer. The resulting mixture was analyzed with a fluorescence spectrophotometer (F-2000, Hitachi High-Technologies Co., Tokyo, Japan; excitation: 390 nm; emission: 470 nm).

Results and Discussion

Effect of Cooling Rate on Surface SH Content of Soymilk Protein Denatured by Heating

Changes in protein surface SH content indicate the making or breaking of disulfide bonds on the surface of protein molecules as well as exposure or concealment of free SH groups. The protein surface SH content of soymilk was determined after heating and cooling (Figure 1). The soymilk sample measured immediately after heating to 80°C, where the sample was cooled to room temperature in an ice bath, showed that the SH content was almost constant. Soymilk measured after cooling at room temperature for 2 h had a decreased surface SH content. However, rapid cooling of heated soymilk at -5°C decreased the SH content to less than slow cooling at room temperature (Figure 1A). Heating at 80°C was found to denature only β-conglycinin and to maintain the inter-polypeptide disulfide bonds of the glycinin subunits as determined by SDS-PAGE (data not shown). The decreased SH content during cooling possibly depended on structural changes in the denatured β-conglycinin. In the case of heating at 100°C, the surface SH content measured immediately after heating decreased, in contrast to the surface SH content observed after heating at 80°C (Figure 1B). This difference between 80°C and 100°C is thought to arise from denaturation of glycinin. The SH content in the soymilk sample cooled at -5°C for 2 h was between that of the other two, namely, the sample immediately after heating and the sample after 2 h of cooling at room temperature. Furthermore, in the case of heating at 115°C, the SH content initially increased, and then decreased after heating was complete. After 2 h of cooling time, the SH content decreased further as in the case of heating at 100°C (Figure 1C). Cooling at -5 °C suppressed this decrease. By heating at temperatures over 90°C, both β-conglycinin and glycinin are expected to undergo denaturation, dissociation to their constituent subunits, and then aggregation (*17*). Successive re-association of dissociated subunits occurs at least partially through SH - SS exchange reactions. This behavior results in decreasing SH content. However, it is thought that rapid cooling inhibits disulfide bond formation among heat denatured proteins, resulting in decreased SH content.

Effect of Cooling Rate on Surface Hydrophobicity of Soymilk Proteins Denatured by Heating

An increase in protein surface hydrophobicity is thought to indicate thermal unfolding of proteins by estimating the exposure of inner hydrophobic region. In the case of heating at 80°C, surface hydrophobicity increased slightly (Figure 2A). The surface hydrophobicity of the soymilk after 2 h of cooling increased more than that measured immediately after heating; however, there was little difference between cooling temperatures at room temperature and -5°C. The surface hydrophobicity of soymilk heated at 100°C increased significantly. In contrast to heating at 80°C, the hydrophobicity decreased after 2 h of cooling. Cooling at -5°C appeared to maintain higher hydrophobicity than cooling at room temperature (Figure 2B). In case of heating at 115°C, similar results were obtained (Figure 2C). The surface hydrophobicity of rapidly cooled soymilk was between that of the other two, also indicating that cooling at -5°C suppressed the decrease in hydrophobicity after 2 h of cooling.

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

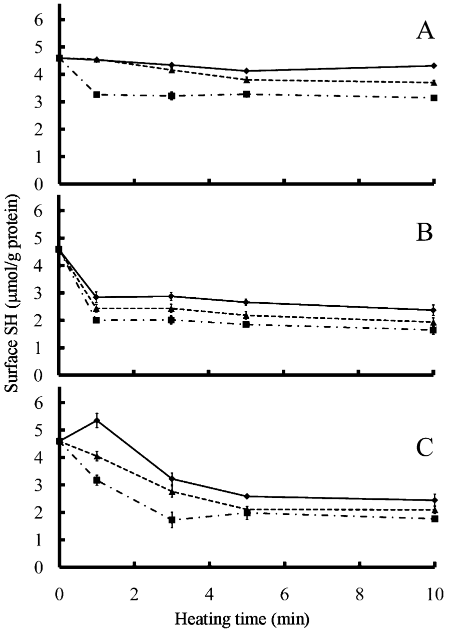


Figure 1. Effect of cooling rate on surface SH content of heated soymilk. A, Soymilk heated at 80°C; B, heated at 100°C; C, heated at 115°C. ♦, Measured Immediately after heating; ▴, after 2h of cooling at -5C; ▪, after 2h of cooling at room temperature.

The protein surface hydrophobicity, which increased upon heating at 100 or 115°C, decreased during cooling for 2 h. This decrease possibly relates to aggregate formation or partial refolding of denatured protein molecules during cooling. Rapid cooling was found to suppress aggregate formation and partial refolding.

In our previous work (*9*, *11*), it was reported that rapid cooling of heated soymilk is essential for gel formation in frozen storage, namely, freeze-gelation. Since coagulate formation is thought to be related to precipitate formation, the effect of cooling rate on the precipitation from heated soymilk was investigated (Figure 3). In the case of heating at 80°C, precipitation was higher than that of the soymilk heated at any other temperatures. Furthermore, it was higher than or comparable with that of the raw soymilk. Slow cooling slightly increased precipitation from refrigerated soymilk. Precipitation decreased with increasing heating temperature. In the case of heating at 100 or 115°C, precipitation from soymilk cooled at -5°C was nearly equivalent to that cooled at room temperature.

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

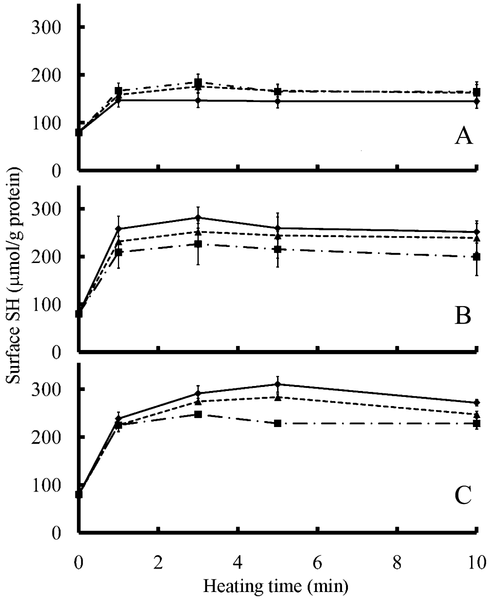


Figure 2. Effect of cooling rate on surface hydrophobicity of heated soymilk. A, Soymilk heated at 80°C; B, heated at 100°C; C, heated at 115°C. ♦, Measured Immediately after heating; ▴, after 2h of cooling at -5°C; ▪, after 2h of cooling at room temperature.

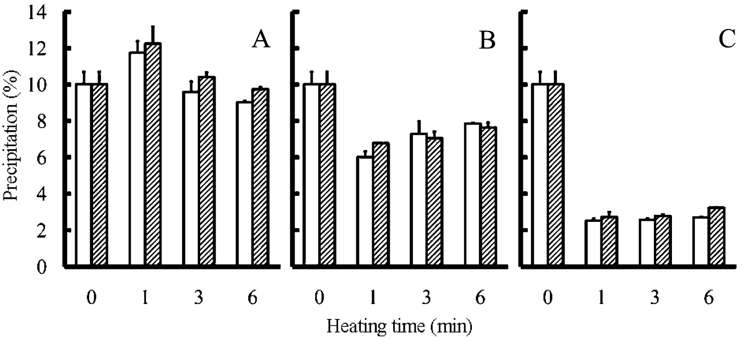


Figure 3. Precipitation of heated and refrigerated soymilk A, Soymilk heated at 80°C; B, heated at 100°C; C, heated at 115°C. Open bar, Soymilk cooled at -5 °C; Hatched bar, cooled at room temperature.

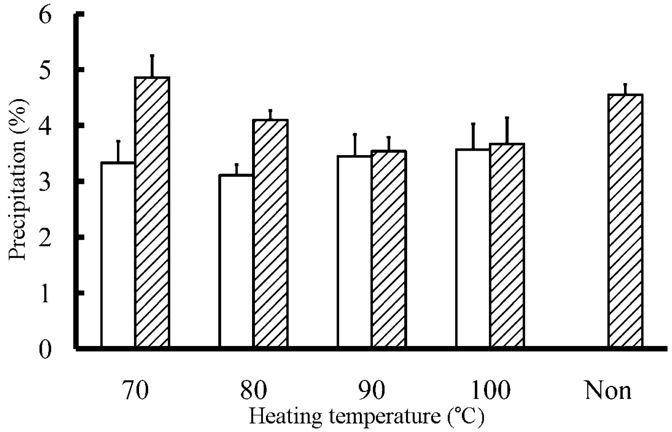


Figure 4. Precipitation of soymilk heated at two temperatures. Open bar, soymilk heated first at lower temperature and successively heated at 115°C; Hatched bar, heated first at 115°C and at lower temperature. Non, non-heated soymilk. (Reproduced from reference (18). Copyright 2008 The Japanese Society for Food Science and Technology.)

Denaturation Behavior of Soymilk Proteins Heated at Two Successive Heating Temperatures

After cooling, the structure of thermally denatured soymilk proteins was altered depending on cooling rates. Regarding the slow cooling as another heating of proteins by residual heat, it is possible for further denaturation to occur, for example, unfolding and/or refolding and aggregating. Thus, we can estimate the effects of two-step heating on the thermal denaturation behavior of the soymilk proteins. The two-step heating was performed as a combination of heating at 115°C in a pressure cooker as a higher temperature and then at 70, 80, 90 or 100°C in a water bath as a lower temperature. Heating first at the higher temperature and then at the lower temperature was carried out in sequence, and the results were compared with those from a reverse sequence, namely two-step heating first at a lower temperature and then at a higher temperature.

Precipitate formation was estimated after the two-step heating (Figure 4). The precipitation from refrigerated soymilk increased due to the heating sequence first at 115°C and then at 70 or 80°C. On the other hand, the opposite heating sequence, namely, heating first at 70 or 80°C and then at 115°C, showed considerably lower precipitation. These results showed that combinations and sequences of heating temperatures affect the properties of heated soymilk, such as precipitation. Therefore, heating sequences as well as individual heating temperatures are important factors for controlling the quality of soymilk.

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

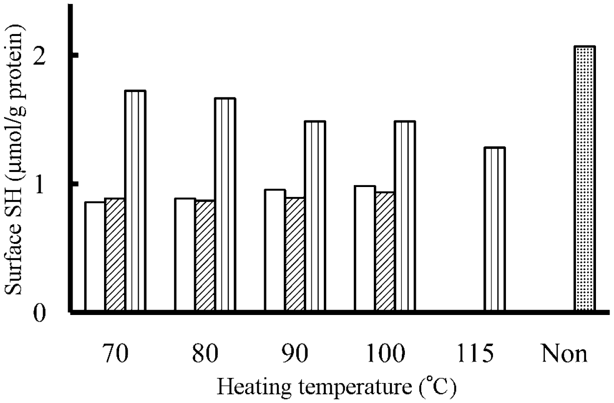


Figure 5. Surface SH content of soymilk heated at two temperatures. Open bar, soymilk heated first at lower temperature and successively heated at 115°C; Hatched bar, heated first at 115°C and at lower temperature; Vertically-striped bar, heated at one temperature. Non, non-heated soymilk. Duplicate measurement was repeated twice. (Reproduced from reference (18). Copyright 2008 The Japanese Society for Food Science and Technology.)

The surface SH content of soymilk proteins was measured during two-step heating (Figure 5). The SH content of proteins in the soymilk heated at two different temperatures was lower than that of the soymilk heated at a single temperature. This decrease in the surface SH content is possibly the result of the extended total heating time. Comparing sequences of heating temperatures, there were no significant differences between heating first at a lower temperature and then at a higher temperature, and heating first at a higher temperature and then at a lower temperature.

Next, the surface hydrophobicity of the soymilk proteins was measured (Figure 6). When 115°C was the higher temperature in the heating sequence, and 90 or 100°C were the lower temperatures, no significant differences were observed, even if the sequence of the heating temperatures was switched. However, the surface hydrophobicity of soymilk heated first at 90°C and second at 115°C was slightly higher than that heated in the reverse order. All temperatures over 90°C were regarded as being essentially equivalent in terms of the heat-induced unfolding of the soymilk proteins, and no significant difference in surface hydrophobicity was observed.

On the other hand, with heating at 70 or 80°C as a lower temperature after initial heating at 115°C, the surface hydrophobicity initially decreased during the second heating step and increased again upon further heating. This decrease during the second heating at 70 or 80°C is attributed to aggregate formation of soymilk protein molecules or partial refolding of the protein molecules that were unfolded during the first heating step. Furthermore, these changes were also thought to relate to the increase in precipitate formation

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

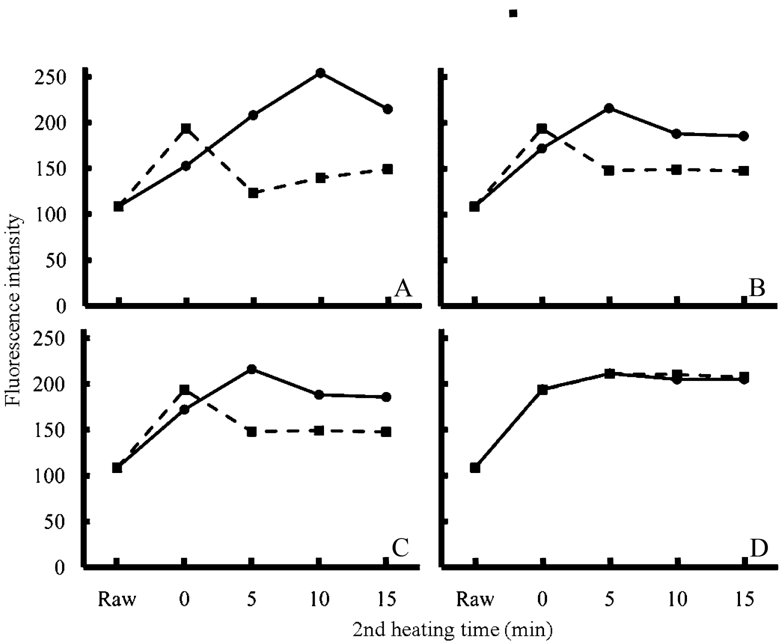


Figure 6. Surface hydrophobicity of soymilk heated at two temperatures. A, soymilk was heated at 70°C (lower temperature) and 115°C (higher temperature); B, heated at 80°C and 115°C; C, heated at 90°C and 115°C; D, heated at 100°C and 115°C. •, soymilk was heated first at lower temperature (70, 80, 90 and 100°C) and successively at 115°C; ▪, heated first at 115°C and successively at lower temperatures. First heating time was 10 min. Duplicate measurement was repeated twice. (Reproduced from reference (18). Copyright 2008 The Japanese Society for Food Science and Technology.)

In conclusion, rapid cooling of heated soymilk suppressed both decreases in the surface SH content and the surface hydrophobicity of soymilk proteins during cooling. Successive heating at two different temperatures affected the denaturation behavior of soymilk proteins, resulting in increasing precipitation of soymilk proteins upon heating at 70 or 80°C as a second heating step after initially heating at 115°C. Since the surface hydrophobicity of the soymilk proteins was initially decreased by the second heating step at 70 or 80°C, this increase in precipitation is possibly related to aggregate formation or partial refolding of the soymilk proteins.

References

1. Lichtenstein, A. H. Soy protein, isoflavones and cardiovascular disease risk. *J. Nutr.* **1998**, *128*, 1589–1592.
2. Chen, J.-R.; Liu, S.-M.; Yang, S.-C.; Suetsuna, K. Soymilk intake is associated with plasma and liver lipid profiles in rats fed a high-cholesterol diet. *Nutrition* **2004**, *20*, 929–933.

3. Messina, M. J. Legumes and soybeans: Overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* **1999**, *70*, 439S–450S.
4. Ishihara, M.; Singh, H.; Chung, G.; Tam, C. Content composition and antioxidative activity of isoflavones in commercial and homemade soymilk and tofu. *J. Sci. Food Agric.* **2007**, *87*, 2844–2852.
5. Ono, T.; Choi, M. R.; Ikeda, A.; Odagiri, S. Changes in the composition and size distribution of soymilk protein particles by heating. *Agric. Biol. Chem.* **1991**, *55*, 2291–2297.
6. Ono, T.; Takeda, M.; Guo, S.-T. Interaction of protein particles with lipids in soybean milk. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1165–1169.
7. Guo, S.-T.; Ono, T.; Mikami, M. Interaction between protein and lipid in soybean milk at elevated temperature. *J. Agric. Food Chem.* **1997**, *45*, 4601–4605.
8. Guo, S.-T.; Ono, T. The role of composition and content of protein particles in soymilk on tofu curding by glucono-δ-lactone or calcium sulfate. *J. Food Sci.* **2005**, *70*, C258–C262.
9. Shimoyamada, M.; Tômatsu, K.; Watanabe, K. Insolubilisation and gelation of heat-frozen soymilk. *J. Sci. Food Agric.* **1999a**, *79*, 253–256.
10. Shimoyamada, M.; Tômatsu, K.; Watanabe, K. Effect of precooling step on formation of soymilk freeze-gel. *Food Sci. Technol. Res.* **1999b**, *5*, 284–288.
11. Shimoyamada, M.; Tômatsu, K.; Oku, S.; Watanabe, K. Interactions among protein molecules in freeze-gel of soymilk and protein structures in heated soymilk during cooling. *J. Agric. Food Chem.* **2000**, *48*, 2775–2779.
12. Hermansson, A.-M. Physico-chemical aspects of soy proteins structure formation. *J. Texture Stud.* **1978**, *9*, 33–58.
13. Zhang, H.; Takenaka, M.; Isobe, S. DSC and electrophoretic studies on soymilk protein denaturation. *J. Therm. Anal. Calorim.* **2004**, *75*, 719–726.
14. Grassetti, D. R.; Muray, J. J. F. The use of 2,2′-dithiobis-(5- nitropyridine) as a selective reagent for the detection of thiols. *J. Chromatogr.* **1969**, *41*, 121–123.
15. Obata, A.; Matsuura, M.; Fukushima, D. Spectrophotometric determination of sulfhydryl groups in soymilk using 2,2′-dithiobis-(5-nitropyridine). *Nippon Shokuhin Kogyo Gakkaishi* **1989**, *36*, 707–711in Japanese.
16. Hayakawa, S.; Nakai, S. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.* **1985**, *50*, 486–491.
17. Petrucceli, S.; Añón, M. C. Thermal aggregation of soy protein isolates. *J. Agric. Food Chem.* **1995**, *43*, 3035–3041.
18. Shimoyamada, M.; Tsushima, N.; Tsuzuki, K.; Asao, H.; Yamauchi, R. Effect of heat treatment on dispersion stability of soymilk and heat denaturation of soymilk protein. *Food Sci. Technol. Res.* **2008**, *14*, 32–38.

Downloaded by UNIV OF GUELPH LIBRARY on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch004

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Chapter 5

Comparison of Laboratory and Industry Methods for the Separation of 7S and 11S Soy Proteins

Xu Jingting, Ren Jianhua, Ye Lingfeng, and Guo Shuntang*

College of Food Science & Nutritional Engineering,
China Agricultural University, Beijing, China
***Corresponding author:**
College of Food Science and Nutritional Engineering, China Agricultural University, No. 17 Qinghua Donglu, Haidian District, Beijing 100083, China.
E-mail: shuntang@cau.edu.cn. Telephone/Fax: +86-10-62737634.

Including our laboratory's isolation techniques (method of Guo and application of rennase from *Mucor pusillus*), this article compared different separation methods of 11S and 7S fractions with industrial and laboratory scales. The principles of most separation techniques are "alkali extraction and acid precipitation" and "cold precipitation" effects. In the laboratory preparation methods, Thanh's method is the first to realize the contemporary separation technique of soy protein components. Nagano's method and Wu's methods are the classical methods quoted for many times. And the Deak's method gives the best isolation efficiency by using Ca^{2+} as a precipitating agent. In the industrial production methods, Wu's method succeeded in the isolation 11S and 7S fraction for the first time. The later researchers have mostly focused on its improvement. The method of Guo is the only method to extract protein in a neutral condition, thus saveing the usage of alkali and avoids the denaturation of protein. Meanwhile, this article pointed out the problems in the current industrial production. Although improvements have been made on the separation process, problems still exist, including complicated procedures, low yields and high costs of production a. Therefore, we futher propose the new direction.

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Introduction

Soy protein, an important vegetable protein, is widely used in the food industry. Two major components of soybean protein are glycinin (11S) and β-conglycinin (7S), which account for approximately 40% and 30% total soybean protein, respectively (*1–3*).

Glycinin is a heterogeneous protein having a molecular weight of 340 to 375 kDa (*4–6*). The structure of the 11S is a hexameric protein composed of several subunits, with each subunit consisting of an acidic (A) and basic (B) polypeptide chain connected by a disulphide linkage (An—S-S—Bn) (*7*). The main body is a stable hexagonal structure of two rings which is formed by the interaction between six acidic subunits (A_1, A_2, A_3, A_4, A_5, A_6) and six basic subunits (B_1, B_2, B_3, B_4, B_5, B_6) (*8*, *9*). Glycinin has the characteristic of "cold precipitation". About 86% glycinin can be precipitated when the defatted soybean protein extracting solution is kept in ice bath (0~2°C) overnight (*10*).

β-conglycinin is a glycoprotein containing 3.8% mannose and 1.2% glucosamine with a molecular weight of 126 to 171 kDa. It is composed of α′, α, and β subunits, which make up seven heterogeneity proteins including B_0(α′ ββ) , B_1 (αββ) , B_2 (α′αβ) , B_3 (α′α′ β) , B_4 (ααα′) , B_5 (ααα) and B_6 (βββ) with subunits linked by hydrophobic and hydrogen bonding (*9*). Although the isoelectric point (pI) of β-conglycinin is pH 4.8-4.9 (*11*), each subunit has its own thermal stability (*12*). By the same token, under the different pH and ionic strength, other characteristics, such as surface hydrophobicity, thermal stability, solubility, thermal cohesion and emulsifying capacity are substantial by different for each β-conglycinin subunit (*12*).

As to physical, chemical and functional properties, 7S and 11S globulins are significantly different in their amino acid compositions and structures. For instance, gel-forming ability and stability of 11S globulin are superior to 7S globulin (*13*). 11S globulin presents superior elasticity, cohesion and extensibility to 7S globulin, and it can form better gel with water-holding capacity (*14*). With the increasing of 11S and 7S ratio, the hardness and viscosity of protein gel increased (*15*, *16*), and emulsifying ability of soy protein is shown an inverse relationship with 11/7S ratio (*17*).

The derivatives of 7S globulin and 11S globulin hydrolysates show special physiological activities. It was reported that glycopeptides hydrolyzed from β-conglycinin are able to modulate the balance of gut microbiota. It inhibited not only the adhesion of pathogenic bacteria to intestinal cells but also the *Salmonella typhimuri*um translocation in Caco-2 epithelial cell monolayers (*18–20*). The 7S globulin hydrolysates are also able to reduce blood cholesterol (*21*, *22*) and plasma triglyceride levels (*23–25*).

Therefore, based on these special physiological activities and functional properties, there is a wide market prospect for 7S and 11S protein fractions in producing functional food or food additives. This paper summarize the researches for 7S and 11S including separating method, product yield, product purity and industrial production, and discuss the industrialization of 7S and 11S separation techniques.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Methods for Separation of 7S and 11S Protein Fractions

The research of 7S and 11S soy globulin separation technique was started in 1960s. Wolf and others utilized the "cold precipitation" effects of 11S to get the crude 11S faction, and then prepared a relatively pure 11S globulin according to the classic method of ammonium sulfate salting-out in 1962 (*26*). However, this method could not be used for separation of 7S globulin at the same time (*27*).

The pI of 11S globulin is pH 6.4, while the pI of 7S globulin is pH 4.8-4.9 (*11*, *28*). With the utilization of the principle that different soybean protein components could precipitate in their isoelectric points, Thanh and other (*29*, *30*) directly separated 11S globulin and 7S globulin by adjusting the pH. Soy storage protein is a kind of alkali-soluble protein. Thus, it can be extracted from the defatted soybean with alkaline solution. Meanwhile, the 7S and 11S fractions can be obtained, respectively, because they are precipitated in different pI. That is the so-called "alkali extraction and acid precipitation"method. It is the first time to separate 7S globulin and 11S globulin simultaneously and laid the foundation for the subsequent separation techniques of the soy protein components.

Existing reports of separation technology indicate the main purpose of separation involves two aspects: one is to study the chemistry and physiological activity of 7S and 11S globulins and the other is to use their special features for food industrial applications. Therefore, in order to meet the requirements for the different uses of isolates, the additives and the technical routes used in the separation process differ. For instance, for the purpose of studying the physiological activity of 7S and 11S globulin, the Tris-HCl buffer was chosen for keeping the native protein structure. Adding the reducing agent such as 2-mercaptoethanol (2-ME) or sodium bisulfite (SBS) in the extracting process to break disulfide bonds between the protein components was for further improving the purity of isolates (*31*). In order to improve the product yield, the inorganic salt is added for the "salting-out" effect, which breaks the electric double layer of protein and promotes the protein precipitation (*32*).

However, the purity requirements of 7S and 11S fraction are not so harsh if they are applied in the food industry. For the industrial production, the principles for choosing separation technique mainly consider cost-saving, high yield, simple separation steps, and the feasibility of industrial production as the principles for the separation. Therefore, this article elaborates the current separation methods of 7S and 11S fractions from the laboratory-scale preparation and pilot-plant-scale production.

Preparation of Laboratory-Scale Methods

Method of Thanh

Thanh and others (*30*) achieved the contemporary separation technique of soy protein foremost. For the sake of stabilizing the reaction system, keeping the native state of proteins and improving the product purity, the protein was extracted with the Tris-HCl buffer with 2-ME. This method is divided into two steps: defatted soybean meal was extracted with 20 volumes 0.03 M Tris buffer

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

(pH 8.0) containing 0.01 M 2-ME at room temperature for 1 h. After insoluble fraction was removed by centrifugation, the extract was adjusted to pH 6.4. The 11s globulin is collected by centrifugating at 2-5°C. The supernatant is adjusted to pH 4.8 with HCl, and 7S glycinin-rich fraction is obtained as the precipitated curd. The precipitated 11S globulin is washed with pH 6.4 0.03M Tris buffer, and then dispersed in the standard buffer (protein content, 2-3%). NaOH solution is added with stirring until the protein was dissolved (pH 7.8). The protein solution is kept at 3-5°C overnight. A trace of precipitate is removed by centrifugation and the supernatant is just the 11S-rich fraction. Meanwhile, 2 N NaOH was added to the 7S globulin precipitate while stirring until the protein dissolves (pH 7.6), then the suspension is adjusted to pH 6.2 with 2 N HCl. After the precipitate was removed by centrifugation, the supernatant is just the 7S-rich fraction.

Due to the first time to realize the contemporary separation technique of soy protein, this method became the classical method and the primary method of extraction and separation of soybean protein components in the laboratory-scale at present.

Methods of Nagano

7S and 11S globulins are both subunit compounds, It is impossible to separate them completely during the separation process. Taking the elimination of cross-contamination between two proteins as the starting point, Nagano and others (*33*) developed a three-step fractionation, discarded the intermediate protein fraction , and improved the purity of 7S fraction. In addition, the method of Nagano modified the method of Thanh includes the following three aspects: (1) Deionized water is replaced by the Tris buffer as the extracted solution, and the pH is adjusted to 7.5; (2) 0.98g/L SBS takes the place of 2-ME as the reducing agent; (3) 0.25mol/L NaCl is added to the supernatant after the 11-S rich fraction is separated with the purpose of increasing the precipitation rate of 7S-rich fraction. It is also a widely used method for laboratory-scale preparation of soy protein components at the moment.

Method of Wu and the Modified Methods

On the basis of Nagano method, Wu et al. (*34*) modified the separation methods of the 7S and 11S fractions, and carried out the pilot-plant fractionation successfully (described in the latter). The only difference with the Nagano's method was the increase of centrifugal speed in three steps (Table 1).

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Table 1. Operating Process for Several Laboratory-sale Methods in Separating 7S and 11S Fracation[a]

operating process			*Nagano (33)*	*Wu (34)*	*Ricket (35)*	*Liu (36)*
Stage 1 extraction		Material	DSF, H_20	○	○	○
		additive	--	--	--	0.03M Tris-HCl
		RML	1:15	○	1:10	○
		pH	7.5	○	8.5	8.5
		Extract time	1h	○	○	2h
		Temp.	Room Temp.	○	45°C	45°C
		Centrifuge	9000g×30min	14300g×30min	Didn't show	○
Stage 2 Separation	Step 1: 11S fraction	Material	Sup1.	○	○	○
		Additive	0.98g/L SBS	○	○	0.01M SBS
		pH	6.4	○	○	○
		Time	overnight	○	--	○
		Temp.	4°C	○	○	○
		Centrifuge	6500g×30min	7500g×20min	○	○
	Step 2: Intermeidiate fraction	Material	Sup2.	○	○	○
		Additive	0.25M NaCl	○	○	○
		pH	5.0	○	○	5.5
		React time	1h	○	○	0.5h
		Temp.	4°C	○	○	○
		Centrifuge	9000g×30min	14300g×30min	○	○
	Step 3: 7S fraction	Material	Sup3.	○	○	○
		Additive	2-fold H_20	○	3-fold H_20	○
		pH	4.8	○	○	○
		Temp.	4°C	○	○	○
		Centrifuge	6500g×20min	7500g×20min	○	○

[a] Nagano, Wu, Ricket, and Liu represents the different methods named by the inventers, respectively; Liu's method extracted protein solution twice and each time was 1 h; "○" represents that the requirement process or condition is identical with the Nagano's method.

Continued on next page.

Table 1. (Continued). Operating Process for Several Laboratory-sale Methods in Separating 7S and 11S Fracation[a]

“—” represents the process or requirement that does not exist; DSF, defatted soybean flake; RML, ratio-of-material to liquid.

As the Wu-based method, Rickert et al. (*35*) studied the separation methods of soy protein in bench-scale and pilot-plant-scale in 2004. The influences of pH, alcohol, reaction temperature and flake-to-water ratios on separation effects were studied, and the parameters and optimized separation processes were determined. Meanwhile, on the basis of Nagao and Thanh, Chun Liu (*36*) researched the optimal method for separation of soy protein from the extracting solution, pH values, temperature, flour/Tris-HCl ratio and the $NaHSO_3$ concentration, and the parameters were also determined according to the final yield and purity of each protein components. The optimized processes of each method are shown in Table 1.

Separation Methods with CO_2 as a Volatile Electrolyte

Traditional soybean-protein-purification techniques of 7S and 11S components often involve the use of inorganic acids to adjust the pH and precipitate the protein. However, when using acidic solutions such as HCl and H_2SO_4 (*37*), the phenomenon of localised excess concentrations of precipitant can occur. And that phenomenon may lead to many problems, such as coprecipitation of other unwanted species, irreversible damage to protein structure, and the formation of many small highly solvated particles. (*38–40*). As a kind of volatile acid, carbon dioxide can be used to acidification of the solution and can be carried out as the electrolyte for soy protein separation technology. In the mean time, the use of carbon dioxide is preferred over the conventional acids because no alkali is required for neutralization. CO_2 can be remixed simply by the release of pressure. It further simplifies the separation technique and reduces the content of ash.

Russell Thiering (*41*) developed a 3-step protein separation method with CO_2 as the acidifier in 1999. In order to enhance the protein yield, intermediate-precipitate fraction was recovered and added to the initial slurry. The whole fraction process was achieved by regulating the extracting protein solution concentration, pressure, reaction temperature, adding volume of CO_2 and acidity of solution. It was found that, the degree of acidification strengthened as the pressure increased, precipitation of the glycinin fraction was the most efficient from highly concentrated soy protein solutions at pH 6.1, and the production of β-conglycinin-rich precipitates was the best from dilute solutions at pH 4.8.

Marijana et al. (*42*) employed CO_2 as a volatile acidifier to prepare the pure 11S component in 2005 as well, and his method is later modified by Russell et al. (*41*). The differences between them were the initial solution and pH for precipitation: Deionized water was used in Russell’s method, while Marijana adopted 30 mM Tris buffer (pH 8.0) with 10 mM 2-mercaptoethanol as the extract solution. The pH for precipitate 11S rich fraction was 6.1 for Russell method,

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

while it was 6.4 for Mraijana's method. However, this method wasonly extracted 11S rich fraction without carrying out the separation research of 7S fraction.

Separation Methods of Ca^{2+} and Mg^{2+} Replace Na^{+} as Precipitating Agent

In the study of separation 7S and 11S fraction, some salt materials were added as the precipitating agent before the step for precipitating 7S globulin. The "salting-out" effect was used to destroy the surface hydration layer and promote the precipitation of 7S. The Na^{+} was used as the precipitating agent mostly, while Ca^{2+}or Mg^{2+} were still used in some case.

Koshiyama (*43*) used 250 mM of $CaCl_2$ to purify the supernatant remaining after cold precipitation of a glycinin-rich fraction. However, the yields and purities of this crude *β*-conglycinin fraction were not determined. Saio and Watanabe (*44*) added 10 mM of $CaCl_2$ to the extraction buffer and defatted soybean meal to first extract a 7S-rich supernatant, and the residue was redissolved and centrifuged to obtain an 11S-rich fraction. The purities on ultracentrifugal basis were about 60%.

Until 2006, on the basis of Wu method, Deak et al. (*45*) studied the separation effects of Ca^{2+} on protein separation. But the calcium salt was not effective in fractionating soy proteins by merely adding it to the fractionation procedure developed by Wu and others (*33*). They changed the pH of precipitate intermediate fraction to pH 6.4, and the separation effect was better than others. The dilution step was no longer employed to obtain the β-conglycinin-rich fraction.

Meanwhile, Deak et al. (*45*) further modified the extraction method for protein solution, brought forward a two-step fractionation, and studied the best concentrations of SO_2 and $CaCl_2$. The optimal process is as below: After the extracted protein supernatant is collected as the method of Wu et al. (*33*), 5 mM SO_2 and 5 mM $CaCl_2$ are added to the solution and pH is adjusted to 6.4 with 2N HCl. The resulting slurry is stored at 4°Cfor 12 to 16 h and centrifuged at 14000×*g* and 4°C for 30 min. An 11S-rich fraction is obtained as the precipitated curd, neutralized, and freeze-dryed. The supernatant is adjusted to pH 4.8 with HCl, stirred for 1 h, and then centrifuged at 14000×*g* and 4°C for 30 min. A 7S-rich fraction is obtained as the precipitated curd. Furthermore, Deak et al. (*46*) carried out the component fractionation research at 4°C referred to as D4C in the original) and 25°C (referred to as DRT in the original) , and discovered the yield and purity of isolated protein were significantly different in the different temperature (*46*). Generally, the yield and purity of protein components produced by this method are better than others.

Zi Teng and others (*47*) made a comprehensive comparison of the protein separation efficiency by using Ca^{2+}or Mg^{2+}, which is based on the two-step fractionation of Deak et al. (*45*). Effective protein separation could be achieved by using 0.5 mM Mg^{2+} as a precipitating agent. 10 mM SBS and 5 mM $MgCl_2$ were added to the protein extracting solution. The resulting slurry was adjusted to pH 6.4 and stirred for 1 h. The 11S-rich fraction was obtained as the precipitated curd after being centrifuged at 3,000g for 15 min, while the supernatant was then adjusted to pH 4.8 with 2 N HCl and stirred for 1 h. After the third centrifugation

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

at 3,000g for 15 min, the precipitated phase was collected as the 7S-rich fraction. Both fractions were redispersed in deionized water and lyophilized.

Application of Enzyme in Components Separation

Protein aggregation was observed when phytate in soybean was hydrolyzed by phytase under certain conditions, and the aggregates consist mostly of glycinin. Based on this charactersistic, Tsutomu Saito and others (*48*) developed a novel method for separating β-conglycinin and glycinin by a phytase treatment. The protein extract, which was extracted in pH 7.5, was adjusted to pH 6.0 with 2M HCl. Phytase was added to this soymilk (1000FYT/100 g of protein in the defatted milk). After that, the resulting mixture was kept for 1 h at 40°C. The solution was then centrifuged to separate the precipitate at the room temperature. The precipitate collected by centrifugation was dispersed in a 4-fold volume of distilled water and neutralized to pH 7.0 with 2 M NaOH (the glycinin fraction). The supernatant was adjusted to pH 5.0 with 2 M HCl and centrifuged. The precipitate was dissolved in a 4-fold volume of distilled water and neutralized to pH 7.0 with 2 M NaOH (the β-conglycinin fraction).

Rennase from *Mucor pusillus* is commonly used in milk curd (*49*, *50*). By using this microorganism in the precipitation of soy protein can precipitate 47% protein, and the aggregate consists mostly of glycinin (*51*). Therefore, our lab developed a technology for separating soybean protein components by using rennase from *Mucor pusillus*. The extracted protein solution was adjusted to pH 5.6 after centrifugation. Rennase at 15U/mL was added and incubated at 60°C for 40 min. The insoluble 11S fraction was obtained by centrifugation at 3600g for 10 min. The supernatant was adjusted to pH 4.5 (2 M HCl), and then centrifuged again at 3000g for 5min. The 7S globulin was obtained as sediment.

Summary of Laboratory-Scale Methods

From the above points of laboratory isolation methods, the operating procedure can be divided into 2-step and 3-step precipitation. The operations are almost similar for 3-step fractionation since the differences of these methods lie in the speed of centrifugation and adding different salts in different concentrations. Using CO_2 as a volatile electrolyte, although the process of adding acid is eliminated, a certain period of time is needed to increase the pressure on the solution, and it is more complex comparatively.

The 2-step fractionation mainly includes the separation method of using $Ca^{2+}$and Mg^{2+} or enzyme as the precipitating agents. The process of “cold precipitation” and the reducing agent were not needed in the enzymatic method. Nevertheless, enzymatic precipitation is relatively cumbersome in the earlier stage and therefore, the demand for the enzyme reaction is strict. Although the author described the method could be used for large-scale preparation, there was no further reports. With the separation methods of using $Ca^{2+}$and Mg^{2+} as precipitating agents were similar with the 3-step fractionation, only omitting the

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

step of removing the intermediate protein fraction. With the relatively simple steps, these methods have some advantages.

Industrial Separation Methods

The Method of Wu and Ricket

The methods of Thanh (*30*) and Nagano (*33*) are all the classical methods for laboratory-scale preparation of 7S and 11S component. However, these two methods are hard to reach the industrial production for the reasons of discontinuous operation, complex process and the use of non-food grade chemical reagents. Wu (*34*) modified the method of Nagano (*33*), developed the industrial separation methods of 7S and 11S components in which the amount of raw material consumption was 20 kg. Wu also changed the pH of basic extract step to 8.5, and reextracted the flake residue for the purpose of improve the protein yield. Though the purity of protein is not high, it is still one of the important references for the technological development of industrial production. The Specific parameters and technological process are shown in Table 2**.**

Based on the above-mentioned method, Wu et al. (*52*) developed the ultrafiltration membrane technology into the industrial separation technique in 2000. For separating 7S component, ultrafiltration and reverse-osmosis membrane technology were operated on the supernatant after extracted 11S globulin. Meanwhile, on the basis of Wu et al. (*34*) and the laboratory-scale (as already described in detail), Ricket et al. (*35*) researched the pilot-plant separation technique and obtained the optimal technology framework. Meanwhile, these two modified methods have the same capacity of handing raw material (20 kg) with the Wu's first method (*34*). The optimal process of each method is shown in Table 2.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Table 2. Operating Process for Several Industrial-sale Methods in Separating 7S and 11S Fracation[a]

Operating process		*Wu (I) (34)*	*Wu(II) (52)*	*Ricket (35)*
Stage 1 extraction	Material	DSF ,H_20	○	○
	RML(1)	1:10	○	○
	RML(2)	1:5	○	--
	pH	8.5	>8.0	○
	Extract time(1)	1h	○	○
	Extract time(2)	0.5h	○	--
	Temp.	20°C	○	45°C

Continued on next page.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Table 2. (Continued). Operating Process for Several Industrial-sale Methods in Separating 7S and 11S Fracation[a]

Operating process			*Wu (I) (34)*	*Wu(II) (52)*	*Ricket (35)*
		Centrifuge	5700rpm	○	9800rpm
Stage 2 Separ-ation	Step 1: 11S fraction	Material	Sup1.	○	○
		Additive	0.98g/L SBS	○	○
		pH	6.4	6.0	○
		Time	overnight	○	○
		Temp.	4°C	7°C	○
		Centrifuge	9800rpm	○	○
		Desalt	RC-30	RC-100	○
		Dry	Spary dry	○	○
	Step 2: Intermediate fraction	Material	Sup2.	--	○
		Additive	0.25M NaCl	--	○
		pH	5.0	--	○
		React time	1h	--	○
		Temp.	5°C	--	--
		Centrifuge	9800rpm	--	○
	Step 3: 7S fraction	Material	Sup3.	○	○
		Additive	2-fold H_20	0.02M SO_2 ; water	3-fold H_20
		pH	4.8	7.0	○
		Temp.	4°C	○	7°C
		time	overnight	--	○
		Centrifuge	9800rpm	--	○
		Desalt	RC-30	--	--
		Concentrate	--	RC-100	--
		Dry	Spray dry	○	○

[a] Wu(I), Wu(II), and Ricket represent the different methods, Wu developed two methods, called Wu (I) and Wu (II), respectively; "○" represents the requirement process or condition that is identical with the first method of Wu. "—"represents that the process or requirement does not exist; RML(1) and RML(2) represent the ratio of material-to-liquid for the first time and second time for protein extraction, respectively. Time (1) and Time (2) represent the extracting time for the first time and second time, respectively; RC-30/RC-100 represents membrane filtration system and a 30KDa/100KDa regenerated cellulose membrane; DSF, defatted soybean flake.

The Method of Guo

In order to fully demonstrate the functional characteristics of soybean protein components and the utilization of 7S and 11S protein components in the actual production, our laboratory studied the separation technique of soybean protein components in a pilot-plant-scale (*53*). The capacity of handing raw material of this method was more than 300 kg.

The steps are as following: defatted soybean meal was dispersed at a 1:12 ratio of flakes to water and stirred the slurry. After the protein was extracted, the insoluble residue was separated by centrifugation with a decanter centrifuges (1800-2000g). The ionic strength and pH values of the extraction were adjusted to 0.05M and 5.5, respectively, and the food grade reducing agent was added to the extraction. With the processes of stirring and keeping the solution for 30 minutes, a decanter centrifuges (1800-2000g) was used for separation of components. The supernatant was just the 7S rich fraction while the precipitation was the 11S rich fraction.

The supernatant was adjusted to pH 4.8 with 2 N HCl and centrifuged (1800g) after holding a slight time, the precipitation was just the 7S rich fraction. Meanwhile, the precipitated glycinin was redissolved in an aqueous solution acidulated to pH 6.4 and centrifuged (1800-2000g) after holding about 30 minutes. The precipitation was the 11S-rich fraction. After that, the precipitated glycinin, β-conglycinin were redissolved in an aqueous solution. Both fractions were centrifuged again for desalting process and neutralized and dried in a spray-dryer.

Summary of Industrial-Scale Methods

The reported pilot-plant-scale for separation techniques were these four methods which were mentioned above. Comparatively, the first method used by Wu (*33*) was more complex, which adopted twice-repeated extraction process for the purpose of improving protein yield. However, it is relatively complicated in the operation process. Only one exaction step was carried out in the method of Ricket (*35*), with simple operating procedure than Wu's method (*34*), but there was a requirement for the extraction temperature in Ricket's method. The second method of Wu (*52*) used was relatively simple, It was separated by two steps, which omit the process of removal intermediate fraction, and the separation of 7S component was carried out by using ultrafiltration membrane so that the efficiency was improved. Generally, the biggest problem of ultrafiltration protein liquid was the membrane contamination. However, this method did not discusse in details about this problem. The 7S-rich fraction and 11S rich-fraction were collected separately before the curd components were separated in the method of Guo. Therefore, the operation is relatively simple and the requirement or equipment is not so high.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Comparison of Separation Effects on Various Separation Methods

According to the available separation techniques, it seems that most methods were based on the principles of "alkali extraction and acid precipitation" and "cold precipitation". However, those fractionations were obviously different in some details, such as separation steps, pH value and precipitating agents, which also leads to variations in the resultant products of 7S and 11S. Subsequently, the effects of various separation techniques on the yield and purity of the product are compared.

Comparison of Separation Effects on Laboratory-Scale Preparation Methods

Because different ways are used to express the isolation efficiency in various articles, for the purpose of comparison, this article takes the yield, protein content and purity of 7S fraction or 11S fraction as the indexes. All the numbers are quoted from the original articles or converted from the original data in the articles.

Table 3. Comparison of separation effects on classical and modified laboratory-scale preparation methods[a]

methods	*11S-rich fraction*			*7S-rich fraction*		
	yield(%)	*protein content(%)*	*purity(%)*	*yield(%)*	*protein content(%)*	*purity(%)*
Thanh (*30*)	15.3	92	78	18.7	91	86
Nagano (*33*)	12	--	>90	6	--	>90
Wu (*34*)	12.9	94.7	95.7	9.8	96.7	77.6
Ricket (*35*)	11.29	96.7	86	9.51	94.04	66
Liu (*36*)	14.4	96.3	92.5	10.7	93.3	95.5

[a] Thanh, Nagano, Wu, Ricket, and Liu represent the different methods named by the inventers.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Table 4. Comparison of separation effects on other laboratory-scale preparation methods[a]

methods		*11S-rich fraction*			*7S-rich fraction*		
		Yield (%)	*protein content (%)*	*Purity (%)*	*Yield (%)*	*Protein content (%)*	*Purity (%)*
Application of Ca^{2+} (*45*, *46*)	modification	12.9	91.8	90.1	15.2	75.8	100
	original	15.5	96	85	23.6	90	81
	D4C	15.5	98.9	81	23.1	90.0	85.6
	DRT	15.7	96.6	71	23.3	91.2	78.6
Application of Mg^2 (*47*)		22.4	--	88.9	16.2	--	81.4
Application of phytase (*48*)		18	88.9	>80	16	78.57	>80
Application of rennase from *Mucor pusillus* (*51*)		19.8	93.4	93	9.5	95.1	88.4
Application of CO_2	Russell (*41*)	28	--	95	4.8	--	80
	Marijanal (*42*)	34	85	97.98	--	--	--

[a] Deak, Russell, and Marijanal represent the different methods named by the inventers.

Table 3 presents the comparation of the classical and modified methods. Thanh's (*30*) method is a 2-step fractionation. The yield of the 7S-rich fraction and 11S-rich fraction are the highest in five methods, and the purity of 7S-rich fraction is relatively high. However, Iwabuchi (*54*) repeated Thanh's method (*30*), and reported that there are about 79% glycinin, 6% β-conglycinin and 15% other components in the11-S rich fraction, whihe 3% glycinin, 45% other protein components and 52% β-conglycinin are in 7S rich fraction. The subsequent researches which commented on the isolation efficiency of Thanh's method were similar to the results of Iwabuchi (*54*).

On the basis of Thanh's method, Nagano developed a new method of 3-step fractionation, which discarded the intermediate protein fraction and improved the purity of 7S globulin. However, the yield of 7S rich fraction was only 6%, which was one third less than Thanh's method because of removing intermediate fraction. The purity of 7S and 11S fractions was greatly improved to more than 90%, respectively..

Wu (*34*), Ricket (*35*) and Liu (*36*) modified the separating conditions and parameters based on the methods of Thanh (*30*) and Nagano (*33*). The yield and purity of each fraction increased markedly. In Liu's method, the yields of 7S-rich fraction and 11S-rich fraction were 14.4% and 10.7%, respectively. Meanwhile, the purity of 7S globulin achieved 95.5% (Table 3).

Aside from the above classical methods and modified methods in laboratory-scale separation method of 7S and 11S globulin, the other separation techniques by using Ca^{2+}, Mg^{2+}, enzyme and CO_2 also produced different isolation efficiencies. The results produced by these methods are summarized in Table 4**.**

Deak (*45*) modified Wu's method Ca^{2+} as a precipitating agent firstly in 2006. The pH value of precipitating intermediate protein fraction was changed to 6.4, and the purity of 7S rich fraction was almost 100%. In the mean time, the author developed a 2-step separation technique by using Ca^{2+}, this method gave the highest yield of 7S-rich fraction in all the current laboratory-scale separation methods. Deak (*46*) further studied the effect of reaction temperature on separation and found low temperature (4℃) could increase the protein content and purity of each fraction due to the effect of "cold precipitation". Comparatively, when Mg^{2+} was used as the precipitating agents, the yield and purity of the 11S-rich fraction was relatively high, up to 22.4% and 80%, respectively.

By the application of phytase, the yield of 11S rich fraction and 7S rich fraction were higher than that of the methods illustrated in Table 3. Meanwhile, the cooling process and a reducing agent are not needed in this method, therefore the processing is simplified and the consumption of resources is saved. As to using the rennase from *Mucor pusillus*, the yield of 11S-rich fraction was relatively high. The11S protein is a stable hexagonal structure formed by the corresponding interactions between six acidic subunits and six basic subunits. Although the pI of glycinin is 6.4, the pI of each subunit is different. The pH values nearby the pI of glycinin probably have a role on precipitating 11S glycinin. The separation method of using the rennase from *Mucor pusillus* was carried out at pH 6.0. Athough it worked for the separation effect, it is hard to conclude whether it is the role of pH value or the enzyme. Hence, in order to explain the mechanism, further studies of applying *Mucor pusillus* in protein separation technique should be carried out.

Another particular method (*41*, *42*) for separating soy protein is using CO_2 as the volatile electrolyte. Using the pressure as a well-controlling process parameter, CO_2 could acidify the solution according to the different requirements. Thus, the protein components could be separated successfully in their own pI. The results of Russell's method (*41*) showed this method was fit for the separation of 11S-rich fraction, the yield and purity of which method (*41*) were 28% and 95%, respectively. As to the 7S-rich fraction, although the yield was lower, the purity was up to 80%. Thus, it is feasible for using CO_2 as the volatile electrolyte in the protein separation technique because the yield and purity of the products can be imporved. Marijana (*42*) further used CO_2 as a volatile electrolyte to separate and purify 11S protein and obtained a high yield and purity of glycinin (34% and 97.98%, respectively). The yield and purity of 11S globulin in this method reached the highest in all the laboratory-scale methods. However, the author did not separate the 7S globulin.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Table 5. Comparison of separation effects on industrialized production technology methods[a]

methods	*11S-rich fraction*			*7S-rich fraction*		
	yield(%)	*protein content(%)*	*purity(%)*	*yield(%)*	*protein content(%)*	*purity(%)*
Wu(I) (*34*)	9.5	91.1	90.6	10.7	97.7	70.4
Wu(II) (*52*)	9.7	91.2	92.8	19.6	91.6	62.6
Ricket (*35*)	11.7	99.8	--	12.25	91.3	68
Guo (*53*)	28.5	94.46	86	5.5	93	65

[a] Wu(I), Wu(II), and Ricket represents the different methods named by the inventers, Wu developed two methods, called Wu(I) and Wu(II), respectively.

Comparison of Separation Effects on Industrialized Production Technology Methods

At present, the researches for the industrial-scale separation technique are mainly four methods illustrated in Table 5. Wu (*34*) studied the separation technique of protein components in pilot-plant-scale for the first time (Wu (I) in Table 5). The yields of 11S-rich fraction and 7S-rich fraction of this method were relatively low (9.5% and 10.7%, respectively). But the purity of 11S globulin was up to 90%.

On the basis of the previous studies, Wu (*52*) simplified the separating procedure of 11S globulin and 7S globulin by the combination of ultrafiltration membrane technique and the principle of pI precipitation (Wu(II) in Table 5). The ultrafiltration membrane was used to separate and purify 7S-rich fraction. In both methods, the yield of 7S-rich fraction in the method 2 was almost twice as that of the method 1, because the intermediate fraction was kept in the supernatant (after removing 11S precipitation fraction), while the purity of 7S rich fraction was only 62.6%, which was caused by the inclusion of the intermediate fraction. However, it is feasible to use method 2 in industrial production because of the simple operating process and the low requirement for purity of the raw material.

Meanwhile, Rickert et al. (*35*) put forward another pilot-plant fractionation technique according to the first method of Wu. This method was relatively simple by keeping the temperature of protein extraction at 45°C and omitting the second extracting step. The yields of 11S and 7S rich fractions were higher than that of the Wu's method (*34*), due to the reason of high temperature could promote the extracting efficiency of protein. The purity of 7S-rich fraction was 68%, which was lower than Wu's method, and the purity of 11S globulin was not reported.

The method of Guo which was put forward by our laboratory is very different from others on the process of separate protein components. The yield of 11S fraction was 2 times higher than Ricket's method and 3 times higher than Wu's method, while the yield of 7S rich fraction was just 5.5%. The purity of 7S component and 11S component were lower than others. It was probably caused by the low-speed centrifugation (4000rpm). Comparatively, because of the high yield of 11S component, it is an effective method for the commercial production.

Discussion on the Industrialized Production

Generally, the separation techniques vary with the different research purposes. Thus, the question is whether each reported pilot-plant-scale production was suitable for industrial production.

Besides the feasibility of industrial production, any method which wants to realize the industrial production has to meet the following requirements as much as possible: (1) low production cost and resource consumption; (2) easy operation; (3) high output; (4) little environmental pollution

Any changes could lead to a great impact on production costs. As to the operation processes of the four methods (Table 5), the first method of Wu (*34*) extracted protein solution in 2-step and obtained the production in 3-step, while Ricket's method (*35*) simplified the process with one extracted protein solution step. Although the second method of Wu carried out a 2-step extracting protein solution procedure, the 2-step fractionation was adopted to simplify the process, thereby saving the costs for the operation.

The material-to-water ratio markedly affects the consumption of resource. Obviously, the more water is used, the more resource is consumed. At this point, Ricket's method (*35*) adopted a smallest material to water ratio (1:10), thus saving the costs correspondingly. Water plays a role of as extraction media in the whole separation process. The discarded waste-water would increase if the water is used too much, and then cause a relatively great environmental pollution problem. Nevertheless, Ricket's method (*35*) put forward a demand of constant temperature for extraction (45°C) and the temperature needs to be reduced quickly after finishing extraction step. From the perspective of industrialization, the treatments of heating or cooling will increase the energy consumption and the energy costs. The precipitation principle of the methods of Wu (*34*, *52*) and Ricket (*35*) was "alkali extraction and acid precipitation". Thus, these methods require a large amount of alkali and acid.

Feasibility is a prerequisite for the realization of industrial production. The method of using ultrafiltration membrane is a simple process with high yield. However, it is easy to cause membrane contamination. Although many researches have been done on membrane technology, it is still hard to be applied in the industrial production due to membrane contamination. On the other hand, Guo's research (*53*) carried out the pilot-plant-scale experiments by the production line in the Tianyuan Group in Gansu province and High Technology Group in Harbin province, respectively. This method meets the needs of industrial production and the results showed it was feasible for commericalization.

In summary, each method has its strengths and weaknesses. Effort should be made to keep its own advantages, reduce the disadvantages, minimize the production cost and improve yield as much as possible for the purpose of realizing the industrial production.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

References

1. Mujoo, R.; Trinh, D. T.; Ng, P. K. W. Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture. *Food Chem.* **2003**, *82*, 265–273.
2. Riblett, R. C.; Herald, T. J.; Schmidt, K. A.; Tilley, K. A. Characterization of β-conglycinin and glycinin soy protein fractions from four selected soybean genotypes. *J. Agric. Food Chem.* **2001**, *49*, 4983–4989.
3. Silvana, P.; Maria, C. A. Soy protein isolate components and their interactions. *J. Agric. Food Chem.* **1995**, *43*, 1762–1767.
4. Iwabuchi, S. Thermal denaturation of *β*-conglycinin. *J. Agric. Food Chem.* **1991**, *39* (1), 27–32.
5. Kitamura, K.; Takagi, T.; Shibasaki, K. Subunit structure of soybean 11S globulin. *Agric. Biol. Chem.* **1976**, *40* (9), 1837–1844.
6. Utsumi, S.; Inaba, H.; Mori, T. Heterogeneity of soybean glycinin. *Phytochemistry* **1981**, *20*, 585–589.
7. Badley, R A.; Atkinson, D.; Hauser, H. The Structure, physical and chemical properties of the soybean protein glycinin. *Biochem. Biophys. Acta* **1975**, *41*, 224–228.
8. Saio, K.; Watanabe, T. Differences in functional properties of soybean proteins. *J. Texture Stud.* **1978**, *9*, 1335–1357.
9. Altschul, A. M.; Wilcke, H. L. New Protein Foods. In *Seed Storage Proteins*; Academic Press, Inc.: 1985; Vol. 5.
10. Yangguo, S.; Li, R. *Technology of Soybean Products*; Light Industry Press, Inc.: Beijing, 1993; p 56.
11. Koshiyama, I. Factors influencing conformation changes in a 7S protein of soybean globulins by ultracentrifugal investigations. *Agric. Biol. Chem.* **1968**, *32*, 879–887.
12. Maruyama, N.; Katsube, Y.; Wada, Y. The role of N-linked glycans and extension regions of soybean β-conglycinin folding, assembly and structural features. *Eur. J. Biochem.* **1998**, *258*, 854–862.
13. Fukushima, D. Recent progress of soybean protein foods: Chemistry. *Technol. Nutr. Food Rev. Int.* **1991**, *7* (3), 323–351.
14. Sen, Y. Y. Formation mechanism of soybean protein gel. *Nippon Nogeikagaku Kaishi* **1988**, *62* (5), 882–885.
15. Zhou, R. B. Structure and fouction of 7S and 11S protein. *J. China Cereals Oils Assoc.* **1998**, *13* (6), 39–42.
16. Chen, H. M.; Hua, Y. F. The study of soy protein compostion and fountion. *China Western Cereals Oils Technol.* **2001**, *26* (3), 36–38.
17. Hu, C.; Huang, L. H.; Li, W. Z. The ratio of 11S/7S globulin impacts soybean protein's functions. *J. China Cereals Oils Assoc* . **2004**, *19* (1), 40–42.
18. Yang, B. C.; Zhang, X. M.; Bao, X. L.; Lv, Y.; Zhang, J.; Guo, S. T. Glycopeptide derived from soybean β- conglycinin inhibits the adhesion of *Escherichia coli* and Salmonella to human intestinal cells. *Food Res. Int.* **2008**, *41*, 594–599.
19. Yang, B. C.; Lv, Y.; Chen, Y.; Wang, J.; Tang, W. X.; Guo, S. T. The inhibitory action of soybean β-conglycinin hydrolysates on *Salmonella typhimurium*

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

translocation in Caco-2 epithelial cell monolayers. *J. Agric. Food Chem.* **2008**, *56*, 7522–7527.

20. Shen, C. L.; Chen, W. H.; Zou, S. X. In vitro and in vivo effects of hydrolysates from conglycinin on intestinal microbial community of mice after *Escherichia coli* infection. *J. Appl. Microbiol.* **2007**, *102* (1), 283–289.
21. Manzoni, C.; Duranti, M.; Eberini, I; Scharnag, H.; Marz, W.; Castiglioni, S.; Lovati, M. R. Subcellular localization of soybean 7S globulin in HepG2 cells and LDL receptor up-regulation by its alpha' constituent subunit. *J. Nutr.* **2003**, *133*, 2149–2155.
22. Duranti, M.; Lovati, M. R.; Dani, V.; Barbiroli, A.; Scarafoni, A.; Castiglioni, S.; Ponzone, C.; Morazzoni, P. The alpha' subunit from soybean 7S globulin lowers plasma lipids and upregulates liver beta-VLDL receptors in rats fed a hypercholesterolemic diet. *J. Nutr.* **2004**, *134*, 1334–13349.
23. Aoyama, T.; Kohno, M.; Saito, T.; Fukui, K.; Takamatsu, K.; Yamamoto, T.; Hashimoto, Y.; Hirotsuka, M.; Kito, M. Reduction by phytate-reduced soybean β-conglycinin of plasma triglyceride levels of young and adult rats. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 1071–1075.
24. Baba, T.; Ueda, A.; Kohno, M.; Fukui, K.; Miyazaki, C.; Hirotsuka, M.; Ishinaga, M. Effects of soybean beta-conglycinin on body fat ration and serum lipid levels in healthy volunteers of female university students. *J. Nutr. Sci. Vitaminol.* **2004**, *50*, 26–31.
25. Moriyama, T.; Kishimoto, K.; Nagai, K.; Urade, R.; Ogawa, T.; Utsumi, S.; Maruyama, N.; Maebuchi, M. Soybean beta-conglycinin diet suppresses serum triglyceride levels in normal and genetically obese mice by induction of beta-oxidation, downregulation of fatty acid synthase, and inhibition of triglyceride absorption. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 352–359.
26. Wolf, W. J.; Babcock, G. E.; Smith, A. K. Purification and stability studies of the 11S component of soybean proteins. *Arch. Biochem. Biophs.* **1962**, *99*, 265–274.
27. Wolf, W. J.; Sly, D. A. Cryoprecipitation of soybean 11S protein. *Cereal Chem.* **1967**, *44*, 653–668.
28. Wolf, W. J. Physical and chemical properties of soybean proteins. *J. Am. Oil Chem. Soc.* **1997**, *54* (2), 112a–117a.
29. Thanh, V. H.; Okubo, K.; Shibasaki, K. Isolation and characterization of the multiple 7S globulins of soybean. *Plant Physiol.* **1975**, *56*, 19–22.
30. Thanh, V. H.; Okubo, K.; Shibasaki, K. Major proteins of soybean seeds: A straightforward fraction and their characterization heterogeneity of beta-conglycinin. *J. Agric. Food Chem.* **1976**, *24*, 1117–1121.
31. Deak, N. A.; Murphy, P. A.; Johnson, L. A. Effects of reducing agent concentration on soy protein fractionation and functionality. *J. Food Sci.* **2006**, *71* (3), C200–C208.
32. Deak, N. A.; Murphy, P. A.; Johnson, L. A. Effects of NaCl concentration influence on β-conglycinin yield and purity during soy protein fractionation. *J. Food Sci.* **2006**, *71* (4), C247–C254.
33. Nagano, T.; Motohiko, H.; Hiroyuki, M.; Kaoru, K.; Katsuyoshi, N. Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. *J. Agric. Food Chem.* **1992**, *48*, 941–944.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

34. Wu, S; Murphy, P. A.; Johnson, L. A.; Fratzke, A. R.; Reuber, M. A. Pilot-plant fractionation of soybean glycinin and β-conglycinin. *J. Am. Oil Chem. Soc.* **1999**, *76*, 285–93.
35. Rickert, D. A.; Johnson, L. A.; Murphy, P. A. Improved fractionation of glycinin and *β*-conglycinin and partitioning of phytochemicals. *J. Agric. Food Chem.* **2004**, *52*, 1726–34.
36. Liu, C.; Wang, H. L.; Cui, Z. M.; He, X. L.; Wang, X. S.; Zeng, X. X.; Ma, H. Optimization of extraction and isolation for 11S and 7S glibulins of soybean seed storage protein. *Food Chem.* **2007**, *102*, 1310–1316.
37. Salt, D. J; Leslie, R. B.; Lillford, P. J.; Dunnill, P. Factors influencing protein structure during acid precipitation: A study of soy proteins. *Eur. J. Appl. Microbiol. Microtechnol.* **1982**, *14*, 144–148.
38. Lillford, P. J.; Wright, D. J. Influence of isoelectric precipitation on the solubility of soybean proteins. *J. Sci. Food Agric.* **1981**, *32*, 315–327.
39. Nash, A. M.; Kwolek, W. F.; Wolf, W. J. Denaturation of soybean proteins by isoelectric precipitation. *Cereal Chem.* **1971**, *48*, 360–368.
40. Wolf, M. Physical and chemical properties of soybean proteins. *J. Am. Oil Chem. Soc.* **1977**, *54*, 112A–117A.
41. Russell, T.; Gerard, H.; Neil, F.; Geert, J. W.; Luuk, V. W. Fractionation of soybean proteins with pressurized carbon dioxide as a volatile electrolyte. *Biotechnol. Bioeng.* **2001**, *73* (1), 1–11.
42. Marijana, G.; van Hateren, S. H.; Ottens, M.; et al. Novel method for the production of pure glycinin from soybean. *J. Agric. Food Chem.* **2005**, *53* (13), 5265–5269.
43. Koshiyama, I. Purification of the 7S component of soybean proteins. *Agric. Biol. Chem.* **1965**, *29*, 885–857.
44. Saio, K; Watanabe, T. Food use of soybean 7S and 11S proteins (extraction and functional properties of their fractions). *J. Food Sci.* **1973**, *38*, 1139–1145.
45. Deak, N. A.; Murphy, P. A.; Johnson, L. A. Soy storageprotein fractionation using Ca^{2+} and $NaHSO_3$. *J. Food Sci.* **2006**, *71*, C413–C424.
46. Deak, N. A.; Murphy, P. A.; Johnson, L. A. Characterization of fractioned soy protein produced by a new simplified proceduce. *J. Am. Oil Chem. Soc.* **2007**, *84*, 137–149.
47. Teng, Z.; Liu, C.; Yang, X. Q.; Li, L.; Tang, C. H.; Jiang, Y. M. Fractionation of soybean globulins using Ca^{2+} and Mg^{2+}: A comparative analysis. *J. Am. Oil Chem. Soc.* **2009**, *86* (5), 409–417.
48. Tsutomu, S.; Mitsutaka, K.; et al. Novel method using phytase for separating soybean β-conglycinin and glycinin. *Biosci., Biotechnol., Biochem.* **2001**, *65* (4), 884–887.
49. Legang, Jin Rennet and its application in cheese production. *China Livestock Products and Food* **1997**, *2*, 78–79.
50. Guo B. H.. *Dairy Chemistry*; Light Industry Press, Inc: Beijing, 2001; p 23.
51. Liu, Z.; Guo, S. T.; Lv, Y. Study on fractionation of soybean protein with rennase from mucor pusillus. *Soybean Sci.* **2008**, *27* (4), 654–658.

52. Wu, S. W.; Patricia, A. M.; Lawrence, A. J.; Mark, A. R.; Alfred, R. F. Simplified process for soybean glycinin and β-conglycinin in fractionation. *Food Chem.* **2000**, *48* (7), 2702–2708.
53. Guo S. T.; Han Y. J.; Meng Y.; Ren C. J.; Li W. Y.. A Method for Extracting Soybean Protein, China Patent 200410029589.4
54. Setsuko, I.; Fumio, Y. Determination of glycinin and β-conglycinin in soybean proteins by immunological methods. *J. Agric. Food Chem.* **1987**, *35*, 200–205.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch005

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

Chapter 6

Estimation of the Mutation Site of a Soyasapogenol A-Deficient Soybean [*Glycine max* (L.) Merr.] by LC-MS/MS Profile Analysis

Hiroko Sasama,[1] Yoshitake Takada,[2] Masao Ishimoto,[3]
Keisuke Kitamura,[4] and Chigen Tsukamoto*,[1]

[1]Graduate School of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[2]National Agricultural Research Center for Tohoku Region, 297 Uenodai, Kariwano, Daisen, Akita 019-2112, Japan
[3]National Agricultural Research Center for Hokkaido Region, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-8555, Japan
[4]Graduate School of Agriculture, Hokkaido University, Kita 9 Nishi 9, Kita, Sapporo, Hokkaido 060-8559, Japan
***Corresponding author:**
Food Chemistry Laboratory Department of Biological Chemistry and Food Science, Faculty of Agriculture, Iwate University, 3-18-8, Ueda, Morioka, Iwate 020-8550, Japan.
Telephone: +81-19-621-6251. Fax: +81-19-621-6251.
E-mail: chigen@iwate-u.ac.jp.

Worldwide attention toward the health benefits from soybean saponins has increased and they have also attracted attention in addressing taste characteristics. There are two soybean saponin aglycons: soyasapogenol A (SA), which causes a bitter astringent taste; and DDMP-conjugated soyasapogenol B (SB), which has health benefits. SA-deficient mutant soybean (Tohoku No. 152) that could have better taste was recently developed. However, the mutation site is unknown therefore undesirable components may accumulate and decrease its food value. Soyasapogenols are thought to be synthesized from 2,3 -oxidosqualene through β-amyrin and hydroxylated by cytochrome P450 family enzymes. If the SA-deficiency were caused by enzyme elimination, intermediate components

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

could accumulate in the mutant seeds. We used LC-MS/MS profile analysis to investigate accumulation of SA precursor components in Tohoku No. 152, but none was detected. We deduced that the SA-deficient mutant eliminates the P450 monooxygenase that hydroxylates the C -21 position of the SA precursor component. SA-deficiency resulted in an increase of DDMP-saponin content.

Keywords: soybean; glycine max; hypocotyls; saponins; soyasapogenols; β-amyrin; cytochrome P450 monooxygenase; *CYP* enzyme; hydroxylase

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

Introduction

Soybean products such as soymilk have always had a hint of a bitter or an astringent aftertaste. Because of this undesirable property, the use of soybeans in the food industry has been limited. If the bitter and astringent aftertastes from soy products were eliminated and health benefits were increased at the same time, the use of soybeans in the food industry could expand. Soybean saponins are known to be the primary cause of the bitter astringent aftertastes (*1*). A better understanding of saponins would allow reduction of undesirable aftertastes and improvement of health benefits (*2–5*). Many scientists have isolated and characterized soybean saponins (*6–13*). The composition of saponins in soybean seeds is quite complex and it is dependent on the soybean variety and on the organ of soybean such as hypocotyls and cotyledon (*14–16*). The variation in saponin composition in soybean seeds is explained by different combinations of genes controlling the sugar chain sequences of soyasapogenol glycosides and the presence of soyasapogenol A (*2*, *17–21*). Therefore it appears to be partially possible to improve saponin components at a genetic level.

There are basically two groups of saponins depending on their chemical structures: group A saponins and DDMP saponins (Figure 1). Group A saponins have soyasapogenol A as the aglycone in the molecule and are detected only in seed hypocotyls. It has 4 hydroxyl groups in the aglycone molecule at the C-3, 21, 22, and 24 positions, and 2 sugar chains are attached at the C-3 and 22 positions. Saponin Ab is a representative saponin of group A. Group A saponins are the primary cause of the bitter astringent aftertaste (*1*, *7*, *8*). Acetylation of the terminal sugar at the C-22 position sugar chain causes the undesirable aftertaste. It is therefore better to eliminate group A saponins from soybeans to increase their value for soy food production. Removal of seed hypocotyls during processing is one practical way to reduce undesirable tastes from soy foods; however, this process discards many health beneficial components from the hypocotyls at the same time. Thus, another way was investigated. DDMP saponins conjugate 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moiety at the C-22 position and a sugar chain at the C-3 position of the soyasapogenol B. Soyasapogenol B does not have the C-21 position hydroxyl group. Saponin βg is a representative saponin of DDMP saponins. Group B and E saponins are derived

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

from the degradation of DDMP saponins during processing for food use (*11–13*). According to some studies, group B and E saponins have a less bitter astringent aftertaste than the group A saponins (*1*). Furthermore, DDMP saponins and their derivatives are expected to have some health benefits in the human colon such as prevention of dietary hypercholesterolemia (*22–24*), suppression of colon cancer cell proliferation (*25*, *26*), and anti-peroxidation of lipids and liver-protecting action by acceleration of secretion of thyroid hormones (*27*). If soybeans lack the group A saponins and have many DDMP saponins in the seed, these soybeans would greatly improve the application of soybeans in the food industry.

Soybean variety Tohoku No. 152 (T-152) is a new variety that is genetically missing its ability to produce soyasapogenol A. T-152, which cannot produce group A saponins, also lacks three lipoxygenase isozymes that produce grassy flavors during soy product preparation. T-152, therefore, is expected to have an excellent potential for soy food production (*28*). Since T-152 was crossed with a wild mutant, there was no information on the mutation site of the soyaspogenol A deficient characteristic. In order to prevent possible side effects from an unknown mutation site, T-152 needed to be investigated further. The purpose of this study is to estimate the mutation site of soyasapogenol A deficient characteristics of T-152 and to clear the saponin components accumulated in the T-152 seeds by using LC-MS/MS profile analysis.

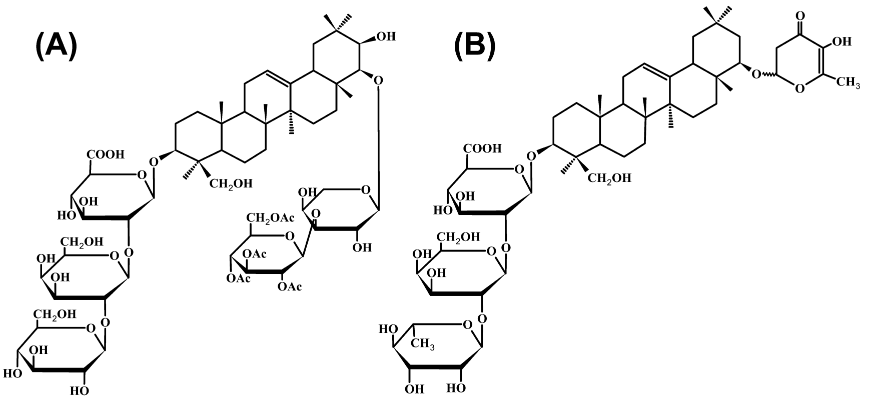


Figure 1. Chemical structures of representative saponin components detected in soybean seed hypocotyls. (A) Saponin Ab (group A saponin); 3-O-[β-D-glucopyranosyl (1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-22-O-[2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl(1→3)-α-L-arabinopyranosyl]-3β,21β,22β,24-tetrahydroxyoleane-12-ene. (B) Saponin βg (DDMP saponin); 3-O-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-22-O-[2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one]-3β,22β,24-trihydroxyoleane-12-ene.

Materials and Methods

Soybeans and Standard Components

Parent varieties, Shirosennari and Tohoku No. 152 (T-152), were grown at the field of National Agricultural Research Center for Tohoku Region and F_1 hybrid seeds were obtained from the cross between them in both 2006 and 2007. F_2 seeds derived from F_1 hybrid plants were obtained in 2007. After drying the parents, F_1, and F_2 seeds harvested in 2007, the hypocotyl part was collected and used in this experiment. Standard components, soyasapogenols A and B were purchased from Koshiro Co. Ltd., Osaka, Japan. β-amyrin was purchased from Extrasynthese, Z.I. Lyon Nord, France. Standard saponins Aa, Ab, Ba, and Bb were gifts courtesy of Dr. Masakazu Shiraiwa, Faculty of Agriculture, Ibaraki University, Japan. Crude soybean saponins were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

Extraction of Saponin Components in Soybean Seed Hypocotyls

Soybean seeds were cut with a hand cutter and the hypocotyls were collected. To extract saponin components for aglycone analysis, a 10-fold volume of aqueous 80% methanol was added to the hypocotyls and extracted saponin components for 12 hours at a room temperature (20 °C) to obtain crude saponin extracts. To extract saponin components for saponin determination, a 50-fold volume of aqueous 80% methanol was added and extracted by the same manner.

Hydrolysis of Saponin Components

Two parts of the crude saponin extract (1.34mL), one part of 12N-hydrochloric acid (0.67mL), and 9 parts of methanol (6.03mL) were mixed in a glass test tube with a screw cap and heated at 75 °C for 2 hours to obtain hydrolysate. After drying the hydrolysate with an evaporator (Rotavapor R-200, Buchi), 1mL of water and 1mL of diethylether were added and subsequently the water layer (lower phase) was removed with a separating funnel. The ether layer was dried and the residue was suspended in 1mL of methanol. After centrifugation at 12,000rpm for 5 min, supernatant obtained (hydrolysate) was used for aglycone analysis.

Aglycone Analysis by LC/PDA/MS

A high performance liquid chromatography with a photo diode array detector and a tandem mass spectrometry (LC/PDA/MS) was performed by using Agilent HP1100 series (Agilent Technologies, Inc., CA, USA) with Brukar Daltonics HCTultra series (Brukar Daltonics, Inc., MA, USA) to analyze aglycone components in the hydrolysate. Monitoring ranges set at wavelength λ=200-360 nm and m/z = 50-1500. The column used was TSK-gel ODS-100V, 150mm x 2.0 mm i.d. (Tosoh Corp, Tokyo, Japan). A gradient condition of acetonitrile was performed from 80 to 100% containing constant 0.1% formic acid for 120 minutes interval. For the first 35 minutes, acetonitrile concentration was kept at 80%, and then it was increased linearly to 100% over the next 40 min, and kept it for 25 min. The last step was reconditioning at 80% for 20 min. The solvent flow rate was 0.15mL/min. To acquire MS and MS/MS data, sample ionization was performed by the positive mode of the atmospheric pressure chemical ionization (APCI) method.

Aglycone Analysis by TLC

To analyze aglycone composition by thin layer chromatography (TLC), an aliquot of each hydrolysate was applied directly to a pre-coated silica gel plate (Kieselgel 60 F-254, Merck) with a glass capillary and developed by using a mixture of benzene: dioxane: acetic acid (20: 5: 1, v/v). Aglycone components were visualized by spraying 10% sulfuric acid and heating at 115 °C for 15 min.

Determination of Saponin Contents

The crude extracts (a 50-fold volume extracts) of F_2 seed hypocotyls were directly used to analyze saponin contents by the LC/PDA/MS. An isocratic elution was performed with acetonitrile: 2-Propanol: water: formic acid = 340: 60: 599:1 (v/v) and the solvent flow rate was 0.15mL/min. The effluent was monitored with a PDA (λ=205-360nm) and positive mode (m/z = 50-1500) of electrospray ionization tandem mass spectrometry (ESI-MS/MS). Total saponin contents (sum of 14 components; Aa, Au, Ae, Ab, Ac, Af, Ba, Bb, Bb′, Bd, Be, αg, βg, and γg) were calculated from standard curve based on the peak area of standard saponin Bb monitored at λ=205nm. The molecular absorbance coefficient for saponin Bb was used to quantify these 14 saponin components.

Results and Discussion

The biosynthetic pathway of soybean saponins has not yet been clearly understood, but it has been estimated that triterpenoid aglycones including soyasapogenol B are synthesized from β-amyrin, which is produced from 2,3-oxidesqualene (Figure 2). β-amyrin is further hydroxylated by some specific P450 cytochrome-monooxygenases, or *CYP* family enzymes. In soyasapogenol B, an oxygen molecule is first introduced to the C-22 position and subsequently onto the C-24 position of the β-amyrin, but soyasapogenol A has an additional hydroxyl group at the C-21 position. Because of these differences, there could be two possible pathways for soyasapogenol A production. Pathway One: oxygen molecule is first introduced at the C-21 position to make 21-hydroxy-β-amyrin. Pathway Two: sophoradiol is produced first and the C-21 position is oxidized to produce cantoniensistriol. In both pathways, oxidization of the C-24 position is thought to be the last step (*29*). If the soyasapogenol A deficiency was controlled by the elimination of the C-24 hydroxylase, the cantoniensistriol should be accumulated in the seeds. In the same way, the 21-hydroxy-β-amyrin should be accumulated by the deficiency of the C-22 hydroxylase. However, no precursor components would be accumulated in the seeds if the C-21 hydroxylase was eliminated, because all of the β-amyrin molecules would flow into the

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

soyasapogenol B production. Therefore, we have assumed that the mutation site of soyasapogenol A deficiency of T-152 would be detected by checking the accumulation of, or lack of precursor components in the seeds. In order to detect the mutation site, we analyzed the presence of precursors by LC-MS/MS profile analysis method.

Figure 3 shows the chemical structures and MS fragment patterns of three standards, soyasapogenol A, soyasapogenol B, and β-amyrin. Their molecular ion peaks $[M+H]^+$, m/z = 475.4, 459.4, and 427.4, respectively, were not detected. However, the fragment ion peaks showed three dehydrations of soyasapogenol A at m/z = 457.5, 439.5, and 421.5, three dehydrations of soyasapogenol B at m/z = 441.4, 423.5, and 405.5, and one dehydration of β-amyrin at m/z = 409.5. These dehydration ion peaks from each molecule correspond to the numbers of hydroxyl groups. Although soyasapogenol A contains four hydroxyl groups, desorption of only one H_2O molecule from the C-21 and C-22 position hydroxyl groups would be possible. Desorption of one H_2O molecule from the C-21 or C-22 position probably generated a compound having a carbon double bond between the C-21 and C-22 position by electron migration. Thus, no proton remained in these positions to dehydrate the residual hydroxyl group. Therefore, soyasapogenol A does not produce fourth dehydration ion peak even though it contains four hydroxyl groups in the molecule. From these data, it was assumed that any saponin precursor components, which have two or three hydroxyl groups in the molecules at the C-3, C-21 and C-22 positions, would be detected by using the annotation profiles of MS analysis. Thus, sophoradiol and 21-hydroxy-β-amyrin $[M+H]^+$ = m/z 443.3 are expected to show the MS fragment at m/z = 425.3 $[M+H-H_2O]^+$ and 407.3 $[M+H-2H_2O]^+$. Cantoniensistriol $[M+H]^+$ = m/z 459.3 will show the MS fragment at m/z = 441.3 $[M+H-H_2O]^+$ and 423.3 $[M+H-2H_2O]^+$.

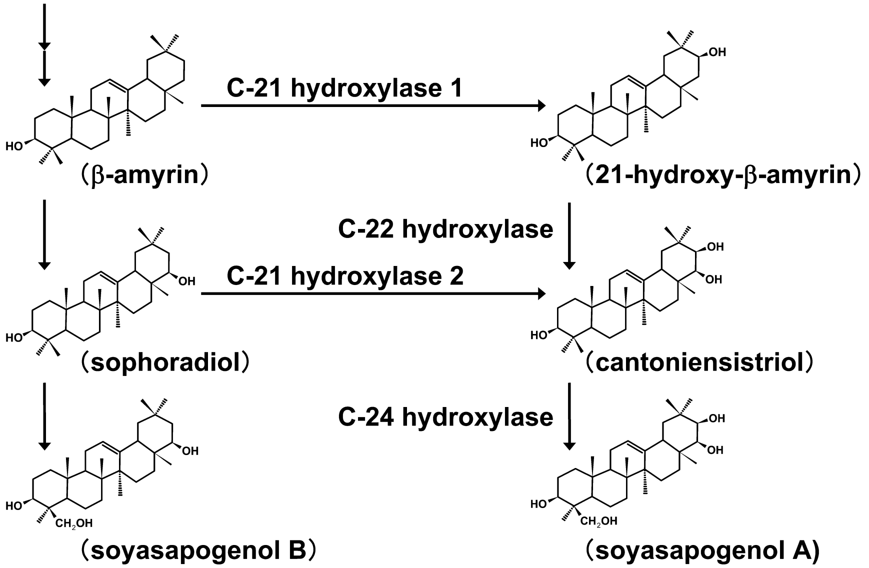


Figure 2. Estimated pathway of soyasapogenols A and B biosynthesis.

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

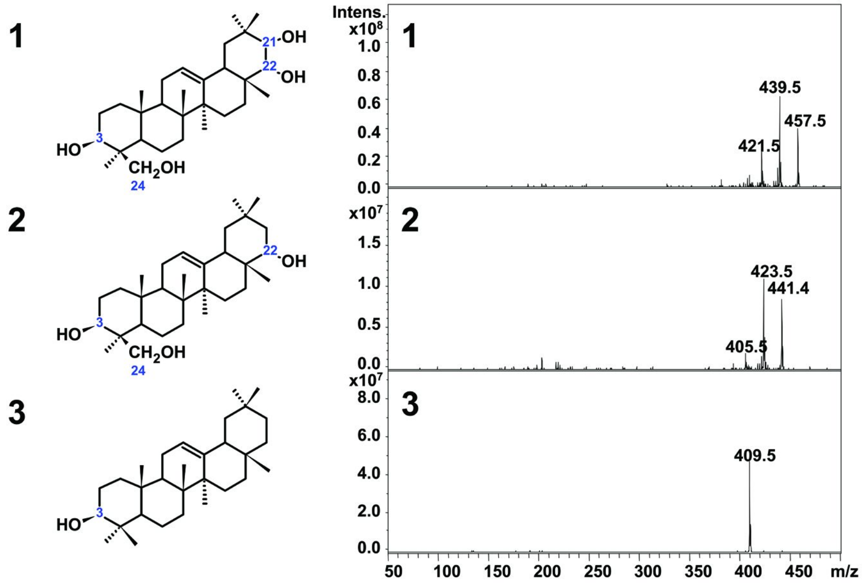


Figure 3. Chemical structures and their MS fragment patterns of soyasapogenol A, soyasapogenol B, and β-amyrin. 1, soyasapogenol A; 2, soyasapogenol B; 3, β-amyrin. As the m/z values presented here were rounded to the nearest tenth, there is rounding errors.

Figure 4-1 shows the HPLC pattern and the retention times of standard components, soyasapogenol A, soyasapogenol B, and β-amyrin. They eluted at 8, 12, and 84 minutes, respectively. Accordingly, any precursor components, which have two or three hydroxyl groups in the molecules, are expected to elute between soyasapogenol A and β-amyrin, which contains four and one hydroxyl groups, respectively. Figure 4-2 shows the HPLC pattern of the hydrolysate of the T-152 extract. Soyasapogenol B was detected and there were two unknown major peaks X1 and X2 at the expected area. The MS fragment patterns of these peak components (Figure 4-A and 4-B), however, showed very different from expected MS patterns corresponding to sophoradiol, 21-hydroxy-β-amyrin, and cantoniensistriol. Therefore, these unknown peaks appear to be non-soyasapogenol precursors. These peak components were detected in the hydrolysate obtained from the hypocotyls of ordinary soybeans. We screened out all components that would have dehydration fragments from the saponin precursors by extraction mode of MS data profile analysis, but no components were identified. The TLC chromatogram of aglycone analysis is shown on Figure 5. The hydrolysate of the T-152 extract showed only soyasapogenols B, and E (*30, 31*) as the main bands and no specific and unique bands were detected in the R_f value between 0.51 (soyasapogenol A) and 0.85 (β-amyrin). From these results, we deduced that T-152 would not accumulate any precursor components of soyasapogenol A. Therefore T-152 would eliminate the P450 (*CYP*) enzyme encoding the C-21 hydroxylase in the seed hypocotyl. If this idea was true, all of the β-amyrin would flow into the soyasapogenol B synthesis and DDMP saponins, which have soyasapogenol B, should be increased. In the next step, we tried to validate this hypothesis.

Saponin contents in the seeds are affected by various factors so we cannot evaluate the hypothesis directly by the quantification of total saponin content of T-152 seeds. We used 213 F_2 seeds derived from F_1 hybrid plants that were obtained from the cross between “Shirosennari”, which was a normal type having the *Sg-5* dominant gene, and “T-152” having the *sg-5* recessive gene to ignore genetic and environmental effects to total saponin contents. F_2 seeds can be divided into two groups according to saponin phenotypes. One group is the *Sg-5* type and it is producing soyasapogenols A and B. Thus, this type contains groups A and DDMP saponin components. The other group is the *sg-5* phenotype and is only producing soyasapogenol B. This type produces only DDMP saponins. Figure 6 shows the total saponin, group A plus DDMP, contents of parent varieties and the frequency distribution of the total saponin contents of the F_2 seed hypocotyls of each saponin type. Female parent Shirosennari contained 3.49 mmol/ 100g and male parent T-152 contained 2.89 mmol/ 100g of the seed hypocotyls at the dry matter base. The average of the *Sg-5* phenotype was 3.47±0.58 mmol/ 100g in average of 162 samples (minimum 2.0, maximum 5.7). The *sg-5* phenotype showed the very same distribution pattern to that of the *Sg-5* type even though the average of 51 samples was 3.11±0.50 mmol/ 100g (minimum 2.0, maximum 4.7). There was no significant difference at 5% level by *t* -test between the two distributions.

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

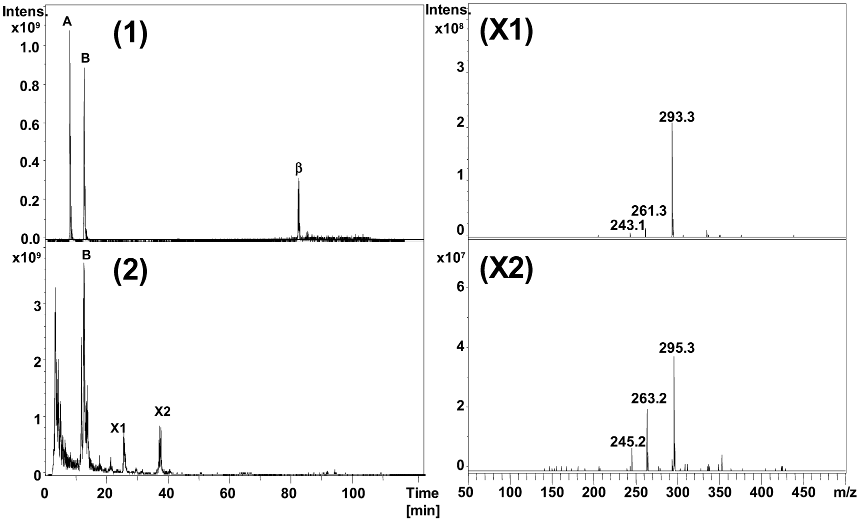


Figure 4. HPLC patterns of standard components and hydrolysate of T-152, and MS fragment patterns of unknown peaks A and B detected in hydrolysate of T-152. (1) HPLC patterns of standard components. A, soyasapogenol A; B, soyasapogenol B; β, β-amyrin. (2) HPLC patterns of hydrolysate of T-152. X1 and X2 show unknown peaks. (A) MS fragment pattern of unknown peak X1. (B) MS fragment pattern of unknown peak X2.

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

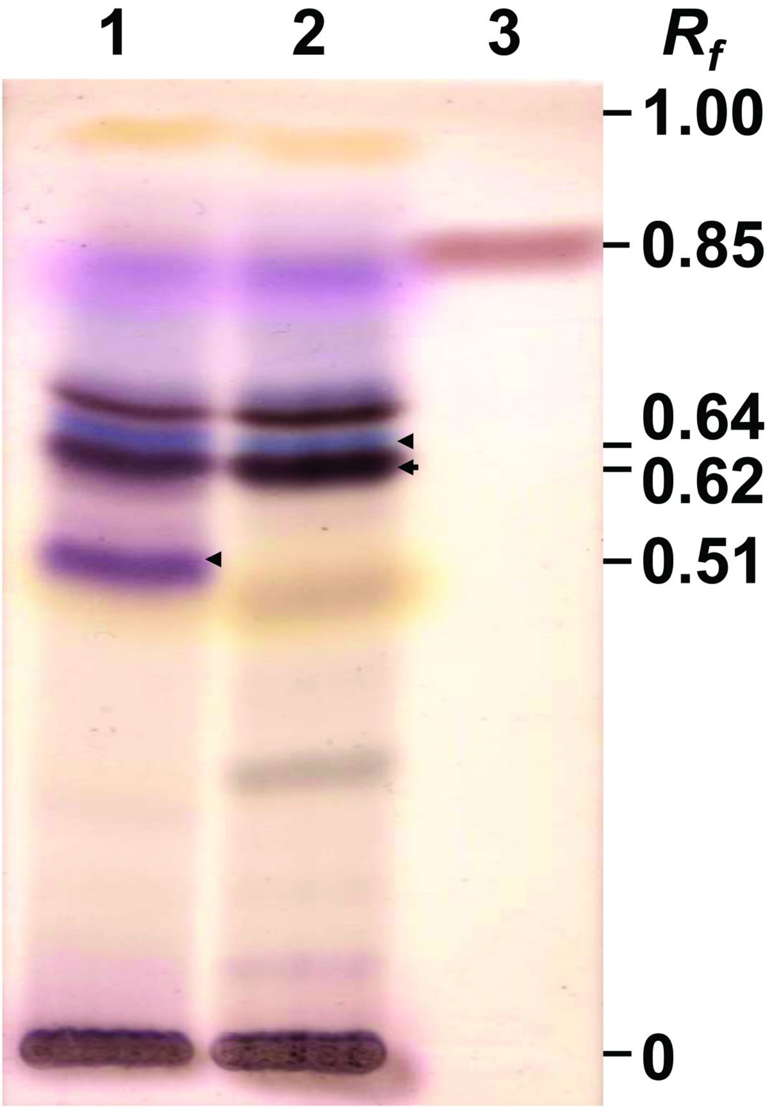


Figure 5. TLC analysis of the hydrolysates of the extracts from the normal variety and T-152. 1. Normal variety; 2. T-152 (soyasapogenol A-deficient variety); 3. β-amyrin (standard).

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

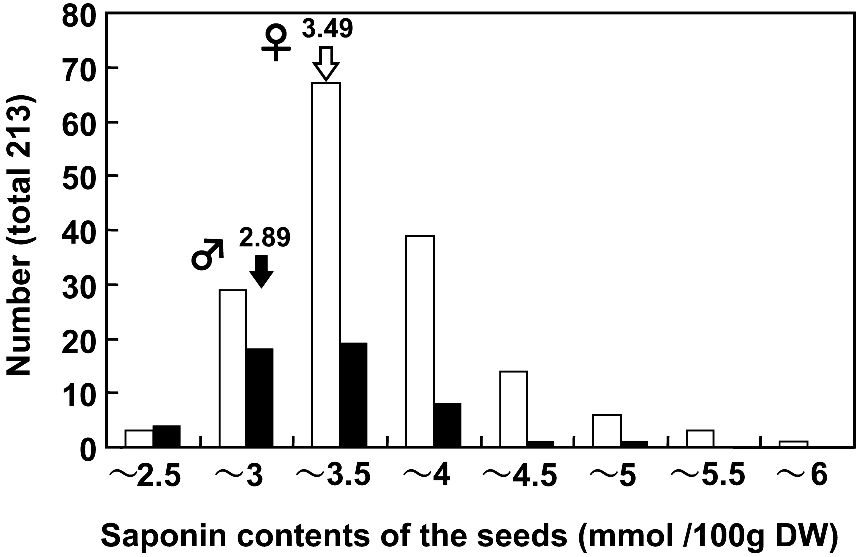


Figure 6. Frequency distribution of total saponin contents of saponin type in the F_2 seed hypocotyls. Total saponin content of a female parent Shirosennari and the Sg-5 phenotypes (n =162) are shown with white bars. A male parent T-152 and the sg-5 phenotypes (n =51) are shown with black bars.

These results strongly suggest that T-152 has no potential to cause side affects and can be a very promising variety for the soy food industry. The elimination of soyasapogenol A will not only improve the flavor by reducing bitter and astringent aftertastes, but will also increase various health benefits from DDMP saponins.

Acknowledgments

This work was partially supported by the Ministry of Agriculture, Forestry, and Fisheries of Japan [Research Project for Utilizing Advanced Technologies in Agriculture, Forestry, and Fisheries (18063)].

References

1. Okubo, K.; Iijima, M.; Kobayashi, Y.; Yoshikoshi, M.; Uchida, T.; Kudou, S. Components responsible for the undesirable taste of soybean seeds. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 99–103.
2. Tsukamoto, C.; Kikuchi, A.; Kudou, S.; Harada, K.; Iwasaki, T.; Okubo, K. Genetic Improvement of Saponin Components in Soybean. In *Food Phytochemicals for Cancer Prevention I: Fruits and Vegetables*; ACS Symposium Series 546; Huang, M. T., Osawa, T., Ho, C. T., Rosen, R. T., Eds.; American Chemical Society: Washington, DC, 1994; pp 372−379.
3. Tsukamoto, C.; Kikuchi, A.; Kitamura, K.; Iwasaki, T.; Okubo, K. Genetic Improvement of Saponin Components in Soybean Seed. In *Soybean Feeds*

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

the World; Chainuvati, C., Sarobol, N., Eds.; Kasetsart University Press: Bangkok, Thailand, 1998; pp 80−85.

4. Tsukamoto, C.; Kikuchi, A.; Takada, Y.; Kono, Y.; Harada, K.; Kitamura, K.; Okubo, K. Genetic Improvement of Soybean Saponins. In *Proceedings of IV International Soybean Processing and Utilization Conference*; Foz do Iguassu: PR, Brazil, 2004; pp 822−829.
5. Tsukamoto, C.; Yoshiki, Y. Soy Saponin. In *Soy in Health and Disease Prevention*; Sugano, M., Ed.; CRC Press, Taylor & Francis Group: New York, 2006; pp 155−172.
6. Kitagawa, I.; Wang, H. K.; Taniyama, T.; Yoshikawa, M. Saponin and sapogenol. XLI. Reinvestigation of the structures of soyasapogenols A, B, and E, oleanene-sapogenols from soybean. Structures of soyasaponins I, II, and III. *Chem. Pharm. Bull.* **1988**, *36*, 153–161.
7. Kitagawa, I.; Taniyama, T.; Nagahama, Y.; Okubo, K.; Yamauchi, F.; Yoshikawa, M. Saponin and sapogenol. XLII. Structures of Acetyl-soyasaponins A_1, A_2, and A_3, astringent partially acetylated bisdesmosides of soyasapogenol A, from American soybean, the seeds of *Glycine max* Merrill. *Chem. Pharm. Bull.* **1988**, *36*, 2819–2828.
8. Taniyama, T.; Nagahama, Y.; Yoshikawa, M.; Kitagawa, I. Saponin and sapogenol. XLIII. Acetyl-soyasaponins A_4, A_5, and A_6, new astringent bisdesmosides of soyasapogenol A, from Japanese soybean, the seeds of *Glycine max* Merrill. *Chem. Pharm. Bull.* **1988**, *36*, 2829–2839.
9. Shiraiwa, M.; Kudou, S.; Shimoyamada, M.; Harada, K.; Okubo, K. Composition and structure of “group A saponin” in soybean seed. *Agric. Biol. Chem.* **1991**, *55*, 315–322.
10. Shiraiwa, M.; Harada, K.; Okubo, K. Composition and structure of “group B saponin” in soy seed. *Agric. Biol. Chem.* **1991**, *55*, 911–917.
11. Kudou, S.; Tonomura, M.; Tsukamoto, C.; Shimoyamada, M.; Uchida, T.; Okubo, K. Isolation and structural elucidation of main genuine soybean saponin BeA. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 142–143.
12. Kudou, S.; Tonomura, M.; Tsukamoto, C.; Uchida, T.; Sakabe, T.; Tamura, N.; Okubo, K. Isolation and structural elucidation of DDMP-conjugated soyasaponins as genuine saponins from soybean seeds. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 546–550.
13. Kudou, S.; Tonomura, M.; Tsukamato, C.; Uchida, T.; Yoshikoshi, M.; Okubo, K. Structural Elucidation and Physiological Properties of Genuine Soybean Saponins. In *Food Phytochemicals for Cancer Prevention I: Fruits and Vegetables*; ACS Symposium Series 546; Huang, M.-T., Osawa, T., Ho, C.-T., Rosen, R. T., Eds.; American Chemical Society: Washington, DC, 1994; pp 340−348.
14. Shimoyamada, M.; Kudou, S.; Okubo, K.; Yamauchi, F.; Harada, K. Distribution of saponin constituents in some varieties of soybean plant. *Agric. Biol. Chem.* **1990**, *54*, 77–81.
15. Shiraiwa, M.; Harada, K.; Okubo, K. Composition and content of saponins in soybean seed according to variety, cultivation year, and maturity. *Agric. Biol. Chem.* **1991**, *55*, 323–331.

16. Tsukamoto, C.; Shimada, S.; Igita, K.; Kuduo, S.; Kokubun, M.; Okubo, K.; Kitamura, K. Factors affecting isoflavone content in soybean seeds: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. *J. Agric. Food Chem.* **1995**, *43*, 1184–1192.
17. Shiraiwa, M.; Yamauchi, F.; Harada, K.; Okubo, K. Inheritance of "group A saponin" in soybean seed. *Agric. Biol. Chem.* **1990**, *54*, 1347–1352.
18. Tsukamoto, C.; Kikuchi, A.; Kudou, S.; Harada, K.; Kitamura, K.; Okubo, K. Group A acetyl saponin-deficient mutant from the wild soybean. *Phytochemicals* **1992**, *31*, 4139–4142.
19. Tsukamoto, C.; Kikuchi, A.; Harada, K.; Kitamura, K.; Okubo, K. Genetic and chemical polymorphisms of saponins in soybean seed. *Phytochemicals* **1993**, *34*, 1351–1356.
20. Tsukamoto, C.; Kikuchi, A.; Shimamoto, Y.; Kim, J. H.; Harada, K.; Kaizuma, N.; Okubo, K. The frequency and distribution of polymorphisms of soybean seed saponins, and identification of a soyasapogenol A-deficient mutant. *Japan. J. Breed.* **1993**, *43* (Supplement 2), 161.
21. Kikuchi, A.; Tsukamoto, C.; Tabuchi, K.; Adachi, T.; Okubo, K. Inheritance and characterization of a null allele for group A acetyl saponins found in a mutant soybean (*Glycine max* (L.) Merrill). *Breed. Sci.* **1999**, *49*, 167–171.
22. Fenwick, G. R.; Price, K. R.; Tsukamoto, C.; Okubo, K. Saponins. In *Toxic Substances in Crop Plants*; D'Mello, J. P. F., Duffus, C. M., and Duffus, J. H., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1991; pp 285−327.
23. Murata, M.; Houdai, T.; Morooka, A.; Matsumori, M.; Oishi, T. Membrane interaction of soyasaponins in association with their antioxidation effect. *Soy Protein Res.* **2005**, *8*, 81–85.
24. Murata, M.; Houdai, T.; Yamamoto, H.; Matsumori, M.; Oishi, T. Membrane interaction of soyasaponins in association with their antioxidation effect: Analysis of biomembrane interaction. *Soy Protein Res.* **2006**, *9*, 82–86.
25. Ellington, A. A.; Berhow, M. A.; Singletary, K. W. Induction of macroautophagy in human colon cancer cells by B-group soybean saponins. *Carcinogenesis* **2005**, *26*, 159–167.
26. Ellington, A. A.; Berhow, M. A.; Singletary, K. W. Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells. *Carcinogenesis* **2006**, *27*, 298–306.
27. Ishii, Y.; Tanizawa, H. Effects of soyasaponins on lipid peroxidation through the secretion of thyroid hormones. *Biol. Pharm. Bull.* **2006**, *29*, 1759–1763.
28. Kikuchi, A.; Sakai, T.; Shimada, H.; Takada, Y.; Kono, Y.; Kato, S.; Yumoto, S.; Shimada, S.; Tabuchi, K.; Tsukamoto, C. Genetic Improvement of Glycosides in Soybean Seed. Abstracts of 10th International Congress of the Society for the Advancement of Breeding Researches in Asia and Oceania, Tsukuba, Japan, 2005; Symposium Session 5, Genetic Diversity of Soybean and Its Use for High Benefit Production, S5-3.
29. Kurosawa, Y.; Takahara, H.; Shiraiwa, M. UDP-glucuronic acid:soyasapogenol glucuronosyltransferase involved in saponin biosynthesis in germinating soybean seeds. *Planta* **2002**, *215*, 620–629.

Downloaded by CORNELL UNIV on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch006

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

Chapter 7

The Interaction of Oil Body and Protein in Soymilk Making

Y. Chen* **and T. Ono**

The United Graduate School of Agricultural Sciences, Iwate University,
Ueda 3, Morioka, Iwate 020-8550, Japan
***chenyeming19821213@gmail.com**

It is known that some protein bind on oil body surface in raw soymilk but they would release from oil body by heating. But the mechanism is not clear. This study showed that protein bind with oil body by electrostatic deposition in raw soymilk. Heating changed intact oil body little but could increase the surface hydrophobicity of protein. It is considered that the surface hydrophobicity increase is the reason for the protein release from oil body which still has a hydrophilic surface after heating.

Soybean [*Glycine max* (L.) Merr.] seeds contain about 20-30% lipid and 30-40% protein. Lipid is mainly stored in an organelle named as oil body while protein is stored in protein storage vacuoles (PSVs). Soybean is soaked, ground, filtrated (raw soymilk) and heated to obtain soymilk. In the grinding process, PSVs are destroyed and their protein (mainly glycinin and β-conglycinin) are dispersed into the homogenate; oil bodies could keep their integrity after short time grinding. After filtration, raw soymilk is obtained. It was reported (*1*, *2*) that some protein bound on the oil body surface and the other were dispersed in raw soymilk; the protein on the oil body surface and that dispersed in raw soymilk, were denatured by heating, dissociated and re-associated to form soluble protein (<40nm) and protein particle (>40nm).

According to the Huang's famous oil body model (*3*, *4*), oil body has a TAGs matrix core, covered by a layer of phospholipids and embedded by oil body intrinsic protein, oleosin. Some surface phospholipids (hydrophilic parts) expose to exterior. Therefore, it is considered that oil body properties are determined by

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

oleosins and the exposed phospholipids. It is known that soybean oil body has three intrinsic oleosins, 24kDa, 18kDa and 17kDa (*5*, *6*). And there is one more protein, known as P34, *Gly* m Bd 30K or *Gly* m Bd 1 (*7*). Initially, it was termed as one kind of oil body oleosins, but in natural seed it was present in PSVs and was one kind of allergy proteins to human (*6*).

As stated above, glycinin and β-conglycinin are deposited in one kind of membrane organelles, PSVs. Also, there are some other proteins (such as lectin, β-amylase) existing in PSVs. PSVs are so weak that they are destroyed by grinding and the deposited protein in them are dispersed into the homogenate. Glycinin has five subunits of A1aB2, A2B1a, A1bB1b, A3B4 and A5A4B3, β-conglycinin has three subunits of α′, α, β. Generally, glycinin exists as hexamer and β-conglycinin exists as trimer. The molecular structures of β homotrimer and A3B4 homohexamer were determined (*8*, *9*) and they had a size of 9.6×9.6×4.4nm and 9.5×9.2×8.0nm by X-ray crystallography, respectively.

In this study, the aim is to clarify the mechanism why protein bind with oil body in raw soymilk and protein release from oil body by heating.

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

Materials and Methods

Materials

Soybeans Tosan 205 (2007), Suzuyutaka (2007) and Yumeminori (2007) were used in this study. Tosan 205 is glycinin deficiency and Yumeminori is α′, α deficiency. Suzuyutaka is a typical soybean in Iwate prefecture, Japan. They were stored at 4°C until use.

Preparation of Oil Body and Protein Fraction from Raw Soymilk and Soymilk

20g soybean was soaked in de-ionized (DI) water for 18h at 4 °C. The total of soybean and DI water was made 200g and ground with an Oster blender (13,900rpm, 2min; Oster, Milwaukee, WI, USA). The homogenate was filtrated through double Kimwipe sheets (Nippon Paper Crecia Co., Ltd. Tokyo, Japan). The filtrate was designated as raw soymilk. Raw soymilk was heated at >95°C for 5min and designated as soymilk.

Sucrose (20% (w/w)) was added into raw soymilk and soymilk. They were stirred and centrifuged (59,860x*g*, 30min). Both raw soymilk and soymilk were separated into three fractions: floating (oil body), supernatant, precipitate. The supernatant fractions were designated as the protein fractions of raw soymilk (native protein, NP) and soymilk (denatured protein, DP). The oil body fractions were washed three times in a solution (20% sucrose, 50mM KCl) by centrifugation (59,860x*g*, 30min). These were designated as oil body of raw soymilk and soymilk.

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

NaCl Effect on Raw Soymilk by Ultracentrifugation (156,000x*g*, 30min)

10ml raw soymilk was put into five beakers, and NaCl was added to make their concentrations of 0mM, 25mM, 50mM, 250mM and 500mM and mixed. 8.8ml of these raw soymilks was added into 10ml centrifuge tube and treated by ultracentrifugation (156,000x*g*, 30min). The picture was taken by a digital camera (FinePix F50fd, Fujifilm holdings, Tokyo, Japan).

Soybean Oil Body Extracted by pH8 Washing (Suzuyutaka)

Sucrose (20%, w/w) was added to raw soymilk (Suzuyutaka). Suzuyutaka was just used here. The raw soymilk was mixed and adjusted to pH 8 by 0.1M KOH. It was treated by centrifugation (59,860x*g*, 30min). The floating was collected and washed two times (59,860x*g*, 30min; pH8, 50mM KCl, 20% sucrose) and used as oil body extracted by pH8 washing.

Preparation of Intact Soybean Oil Body

20g soybean was soaked in 30mM Tris for 18h at 4°C. The total soybean and 30mM Tris was made to 200g. Then it was ground for 2min and filtrated. 20% sucrose was added, stirred for 20min and centrifuged (59,860x*g*, 30min). The floating fraction (oil body) was obtained and washed 4 times by centrifugation (59,860x*g*, 30min; 30mM Tris, 20% sucrose). The floating fraction was carefully resolved into 30ml DI water with a stirrer. The oil body suspension was dialyzed in DI water by 1:10000. This was designated as intact oil body suspension.

Oil Bodies of Four Mixtures

Four mixtures were prepared from 5ml unheated intact oil body (UOB) suspension and 20ml protein fraction of raw soymilk (NP), 5ml heated intact oil body (HOB) suspension (>95°C, 5min) and 20ml NP, 20ml UOB and 5ml protein fraction of soymilk (DP), and 20ml HOB and 5ml DP. They were stirred and centrifuged (59,860x*g*, 30min). Their floating fractions were obtained and washed three times by centrifugation (59,860x*g*, 30min; 50mM KCl, 20% sucrose). These were termed as oil bodies of four mixtures.

SDS-PAGE

SDS-PAGE was conducted with the method by Laemmli (*10*) with the concentrations of the stacking and running gels being 5% and 12.5%, respectively. The buffer in the reservoir contained 0.025M Tris, 0.192M glycine and 0.1% SDS, while the buffers in the stacking and running gels were 0.125M Tris-HCl (pH 6.8) and 0.38M Tris-HCl (pH8.8), respectively. 0.01% samples contained 0.25M Tris-HCl (pH 6.8), 1% SDS, 2% 2-mercaptoethanol, glycerol and bromophenol blue. This stood for overnight. Each sample was put into a sample well in the stacking gel and electrophoresed.

Coomassie brilliant blue G-250 was used for staining protein in gel by the method of Blakesley and Boezi (*11*). Gel was destained using tap water and dried on a filter paper (No. 2, Advantec Toyo Co., Tokyo Japan) in decompression condition at 75°C.

pH and $CaCl_2$ Effects on Unheated and Heated Intact Oil Body

1ml intact oil body suspension was added into six test tubes and diluted 10 times with DI water and adjusted to pH 5.0, 5.2, 5.4, 5.6, 5.8 and 6.0 with 0.01M HCl solution, respectively. They were centrifuged at 400x*g* for 10min.

1ml intact oil body suspension was added to six test tubes and diluted 10 times with DI water and prepared to $CaCl_2$ 0mM, 5mM, 10mM, 15mM, 20mM and 25mM. They were centrifuged at 1,600x*g* for 20min. The pictures were taken by the digital camera above.

Surface Hydrophobicities of Intact Oil Body and Protein

1ml intact oil body solution was diluted to 40 times with 0.1M phosphate buffer (pH6.8). 20ml suspension was heated at >95°C for 5min. Six samples of 2ml diluted heated oil body suspensions were adjusted to 0, 2, 4, 6, 8 and 10×10^{-5}M of 1-anilino-8-naphtalene sulfonate (ANS) with a final volume of 10ml by using the 0.1M phosphate buffer. The six samples of unheated diluted oil body suspensions were prepared in the same way. The fluorescence intensity was determined after 2h by a fluorescence spectrophotometer (RF-5300PC, Shimadzu Co., Tokyo, Japan). The excitation wavelength was 375nm and the fluorescence wavelength was 475nm.

1ml protein fraction of raw soymilk (NP, Tozan 205) was diluted with 0.1M phosphate buffer (pH6.8). 20ml suspension was heated at >95°C for 5min. Then it was done just as the intact oil body.

Results and Discussion

The Interactions between Oil Body and β-Conglycinin or Glycinin

It was reported (*2*) that protein (mainly β-conglycinin and glycinin) bound with oil body in raw soymilk and protein released from oil body by heating. But the respective interactions between oil body and glycinin or β-conglycinin are not clear. Thus, Tosan 205 (glycinin deficiency) and Yumeminori (α′ and α deficiency) were used to examine them. The NaCl effect on raw soymilk was examined by ultracentrifugation (156,000x*g*, 30min). Figure 1 shows that there are little floating fraction and much precipitate in low concentration (0-50mM; 1-3) NaCl, floating fraction appear and precipitate decreases in high concentration (250-500mM; 4-5) NaCl Yumeminori raw soymilk; there are floating fractions in all (0-500mM; 6-10) Tosan 205 raw soymilk.

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

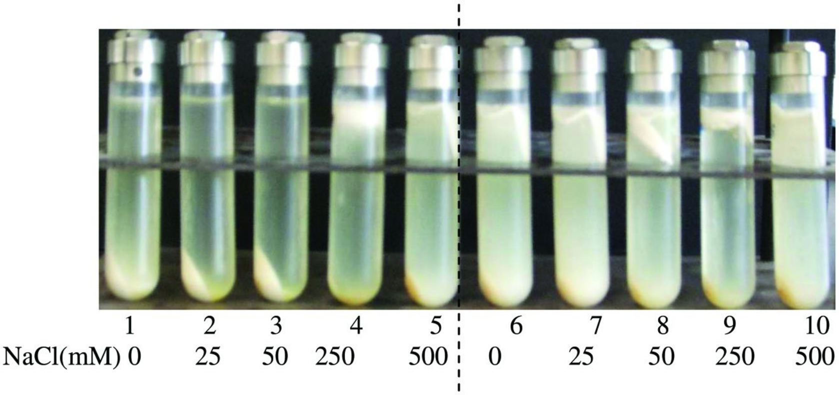


Figure 1. The NaCl (0-500mM) effect on raw soymilk by ultracentrifugation (156,000xg, 30min). Centrifuge tubes 1-5 (Yumeminori raw soymilk) and 6-10 (Tozan 205 raw soymilk): 1 and 6, 0mM; 2 and 7, 25mM; 3 and 8, 50mM; 4 and 9, 250mM; 5 and 10, 500mM NaCl.

The β homotrimer and A3B4 homohexamer have a size of 9.6×9.6×4.4nm and 9.5×9.2×8.0nm, respectively (*8*, *9*); the average size of soybean oil body is about 380nm (*12*). By calculation, the oil body density would be smaller than water (1g/cm^3) if oil body is covered by just one layer of glycinin or β-conglycinin (protein, 1.2g/cm^3; oil, 0.92g/cm^3). Therefore, it is considered that oil body in Yumeminori raw soymilk is covered by many layers of glycinin molecules while that in Tosan 205 raw soymilk is covered by one layer of β-conglycinin. This should be caused by the different properties of glycinin and β-conglycinin. It is known (*13*) that pH 6.4 is used for glycinin isolation from β-conglycinin. This means that glycinin has little solubility at pH 6.4. So glycinin should have a trend to aggregate in raw soymilk (about pH6.5). This should be the reason why oil body could be covered by many layers of glycinin in Yumeminori raw soymilk. β-conglycinin (*14*) is one kind of glycoprotein and its subunits, especially α′ and α, have two oligosaccharide chains and a highly hydrophilic extension region (β have one oligosaccharide chain but no extension region). This should be the reason why Tosan 205 oil body is just covered by one layer of β-conglycinin.

The Molecular Interactions between Oil Body and Protein

The result above showed that oil body could be covered by many layers of glycinin or one layer of β-conglycinin. But the molecular interactions between oil body and protein (glycinin and β-conglycinin) are not clear. Thus, oil body was extracted from Suzuyutaka raw soymilk by pH8 washing. Figure 2 shows that raw soymilk oil body (lane 3) not only contains the oleosins (24kDa, 18kDa and 17kDa) but also β-conglycinin and glycinin while oil body extracted by pH 8 (lane 4) does not contain glycinin and β-conglycinin. It was reported (*15*) that protein could bind with cell membrane by electrostatic deposition or hydrogen bond. Oil body has a similar surface (protein and phospholipid) with cell membrane.

Thus, it is considered that both β-conglycinin and glycinin bind with oil body by electrostatic deposition (Figure 2).

Heating Effects on Intact Soybean Oil Body

From this section, we want to clarify the mechanism for the protein release from oil body by heating. It is known that soybean protein (glycinin and β-conglycinin) would be denatured by heating. But the heating effect on oil body is not known. Thus, intact oil body was extracted by 30mM Tris washing. At first, the heating effects on surface charge were examined. As shown in Figure 3, unheated (a) and heated (b) intact oil body show the same trend, both of them coagulate at pH5.4-5.8. Tzen *et al.* has reported (*16*) that seed oil bodies from diverse species have an isoelectric point in pH range 5.7-6.6. Thus, it is suggested that intact soybean oil body has an isoelectric point in pH 5.4-5.8 and holds negative charge in soymilk (about pH6.5).

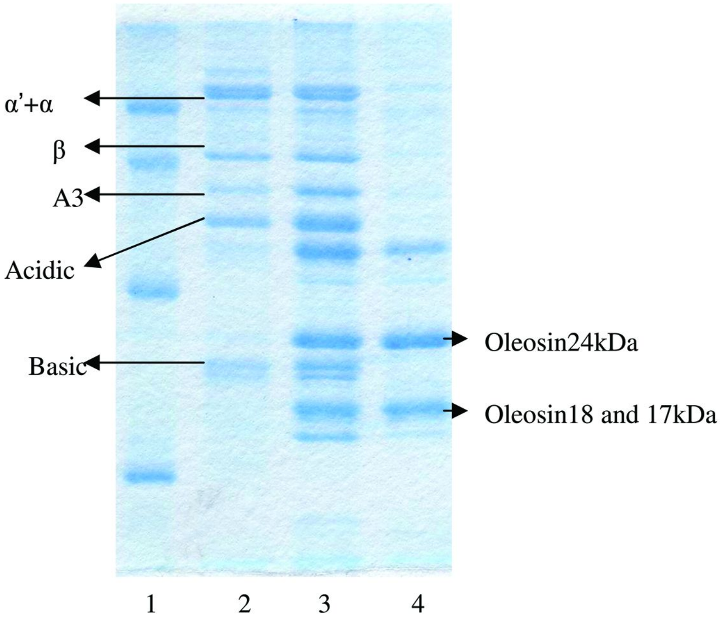


Figure 2. The SDS-PAGE pattern of the protein compositions of soybean oil body extracted by pH 8 washing. Lane 1, marker; Lane 2, raw soymilk; Lane 3, raw soymilk oil body; Lane 4, soybean oil body extracted by pH8 washing. Marker: Bovine serum albumin (67kDa), Ovalbumin (45kDa), Chymotrypsinogen (25kDa), Lysozyme (14kDa).

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

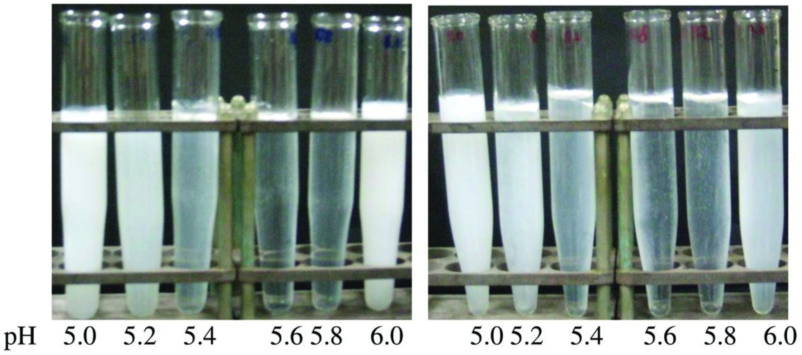


Figure 3. The pH effects on unheated (a) and heated (>95°C, 5min; b) intact soybean oil body.

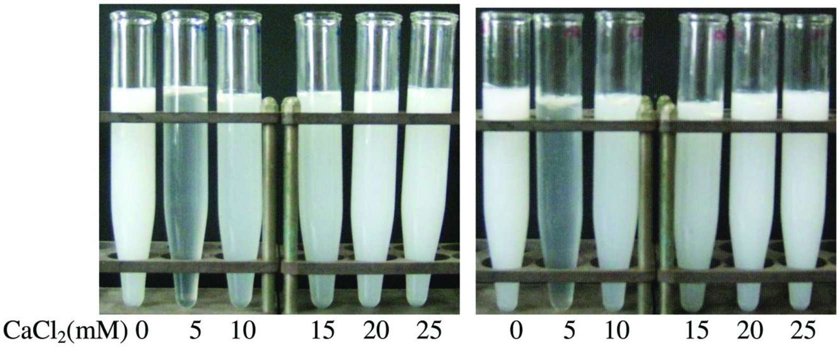


Figure 4. The $CaCl_2$ effects on unheated (a) and heated (>95°C, 5min; b) intact soybean oil body.

Calcium ion (Ca^{2+}) is used as a kind of coagulant for tofu making. Its effect on intact oil body is not clear. Figure 4 shows that unheated and heated intact oil body show the same trend. Intact oil body does not coagulate when $CaCl_2$ is 0mM, but heavily coagulates (1,600x*g*, 20min) when $CaCl_2$ is 5mM. Then coagulation is gradually weakened. It is considered that the intrinsic negative charge of intact oil body is why intact oil body does not coagulate at 0mM $CaCl_2$; Ca^{2+} plays a role of Ca-bridge when Ca^{2+}concentration is low (5mM), but intact oil body would change to have positive charge when Ca^{2+}concentration is high, which makes intact oil body disperse again.

The results above showed that heating did not give obvious effect on the surface charge of intact oil body. But it is well known that the surface hydrophobilicities of many proteins increase by heating owing to the expose of hydrophobic amino acid to the exterior. As stated above, there are oleosins on oil body surface. Therefore, the heating effect on the surface hydrophobicity of intact oil body was examined. Figure 5 shows that the surface hydrophobicity of intact oil body is not affected but that of native soybean protein (NP, protein fraction of raw soymilk (Tosan 205)) is increased by heating.

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

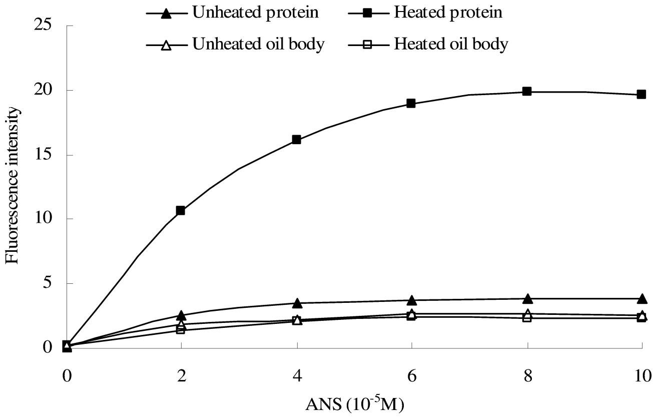


Figure 5. The surface hydrophobicities of unheated and heated intact soybean oil body and protein fraction of raw soymilk (Tosan 205).

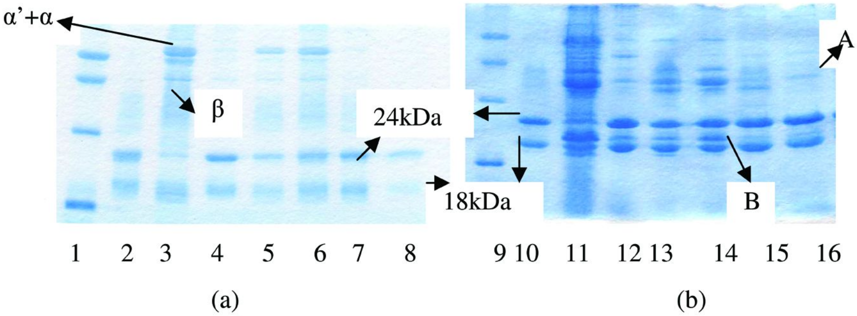


Figure 6. The heating effect on the interaction between intact oil body and protein (a, Tosan 205; b, Yumeminori). Lanes 1 and 9, marker; lanes 2 and 10, intact oil body; lanes 3 and 11, raw soymilk oil body; lanes 4 and 12, soymilk oil body; lanes 5 and 13, oil body from unheated intact oil body (UOB) and protein fraction of raw soymilk (NP) mixture; lanes 6 and 14, oil body from heated intact oil body (HOB) and NP mixture; lanes 7 and 15, oil body from UOB and protein fraction of soymilk (DP) mixture; lanes 8 and 16, oil body from HOB and DP mixture. Marker: Bovine serum albumin (67kDa), Ovalbumin (45kDa), Chymotrypsinogen (25kDa), Lysozyme (14kDa).

Heating Effect on the Interaction between Intact Oil Body and Protein

The results above showed that the surface charge and hydrophobicity of intact oil body were not affected by heating. This means that intact oil body might change little by heating. Therefore, the mechanism for protein release from oil body might be induced by protein denaturation. In order to clarify it, four mixtures were prepared: unheated intact oil body (UOB) and protein fraction of raw soymilk (NP), heated intact oil body (HOB) and NP, UOB and protein fraction of soymilk (DP), HOB and DP. Their oil bodies were prepared by centrifugation. Figure

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

6(a) shows intact oil body from Tozan 205 only has two bands (oleosins 24kDa, 18kDa). 17kDa should be included in the band 18kDa. Other than oleosin bands, raw soymilk oil body has α′, α, β bands but soymilk oil body contains little α′, α, β bands. Interestingly, UOB and HOB could be bound by β-conglycinin (lanes 5 and 6) but they could not be bound by denatured one (lanes 7 and 8). Yumeminori (Figure 6(b)) shows the same trend. These reveal that heating changes intact oil body little and the protein release from oil body is induced by the heat denaturation of soybean protein (mainly glycinin and β-conglycinin).

Figure 5 show that the surface hydrophobicity of soybean protein is increased by heating. Huang reported (*4*) that oil body has a hydrophilic and negatively charged surface at neutral pH. According to the result above (mainly Figure 5), intact oil body should still have a hydrophilic surface after heating. Thus, the hydrophilic surface of intact oil body should be the reason why heat-denatured protein (increased surface hydrophobicity) release from oil body.

Conclusion

Lipid and protein locate in different organelles with lipid in oil bodies and most protein in protein storage vacuoles (PSVs). So oil body and protein could not freely interact with each other in soybean seed. By soaking, PSVs absorb water and become expanded beautiful sphere (picture is not shown) while oil body could not absorb water. By grinding (13,900rpm, 2min), oil body keeps the integrity while PSVs are destroyed and protein release into the homogenate. Generally, raw soymilk is about pH 6.5, where protein have negative charge but weak, especially glycinin. As a result, protein bind with oil body by electrostatic deposition. In addition, glycinin not only could bind with oil bodies but also could bind with each other, which causes the formation of large oil body and glycinin aggregates. β-conglycinin (glycoprotein) is different from glycinin and just could form one layer on oil body surface by electrostatic deposition.

Heating changes intact oil body little and still hold hydrophilic surface. On the other hand, raw soymilk protein change to protein particles and soluble protein by heating. These protein particles and soluble protein are more surface hydrophobic than raw soymilk protein. The two things above cause the protein release from oil body.

References

1. Ono, T.; Murayama, T.; Kaketa, S.; Odagiri, S. *Agric. Bio. Chem.* **1991**, *55*, 2291–2297.
2. Guo, S. T.; Ono, T.; Mikami, M. *J. Agric. Food Chem.* **1997**, *45*, 4601–4605.
3. Tzen, J. T. C.; Huang, A. H. *J. Cell Biol.* **1992**, *117*, 327–335.
4. Huang, A. H. C. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 177–200.
5. Herman, E. M. *Planta* **1987**, *172*, 336–345.
6. Kalinski, A.; Melroy, L. D.; Dwivedi, S. R.; Herman, M. E. *J. Bio. Chem.* **1992**, *267*, 12068–12076.

7. Wilson, S.; Blaschek, K.; de Mejia, E. G. *Nutr. Rev.* **2005**, *63*, 47–58.
8. Maruyama, N.; Adachi, M.; Takahashi, K.; Yagasaki, K.; Kohno, M.; Takenaka, Y.; Okuda, E.; Nakagawa, S.; Mikami, B.; Utsumi, S. *Eur. J. Biochem.* **2001**, *268*, 3595–3604.
9. Adachi, M.; Kanamori, J.; Masuda, T.; Yagasaki, K.; Kitamura, K.; Mikami, B.; Utsumi, S. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7395–7400.
10. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
11. Blakesley, R. W.; Boezi, J. A. *Anal. Biochem.* **1997**, *82*, 580–582.
12. Chen, Y.; Yamaguchi, S.; Ono, T. *J. Agric. Food Chem.* **2009**, *57*, 3831–3836.
13. Thanh, V. H.; Shibasaki, K. *J. Agric. Food Chem.* **1976**, *24*, 1117–1121.
14. Maruyama, N.; Katsube, T.; Wada, Y.; Oh, M. H.; Barba de la rosa, A. P.; Okuda, E.; Nakagawa, S.; Utsumi, S. *Eur. J. Biochem.* **1998**, *258*, 854–862.
15. Taiz, L.; Zeiger, E. *Plant Physiology*, 4th ed.; Sinauer Associates, Inc.: Sunderland, MA, 2006; p 6.
16. Tzen, J. T. C.; Cao, Y. Z.; Laurent, P.; Ratnayake, C.; Huang, A. H. C. *Plant Physiol.* **1993**, *101*, 267–276.

Downloaded by OHIO STATE UNIV LIBRARIES on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch007

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Chapter 8

Storage-Induced Color and Biochemical Changes of Soybeans As Related to Soymilk and Tofu Making

Sam K. C. Chang[*]

**Department of Cereal and Food Sciences, IACC 322. Dept. 2710, Box 6050,
North Dakota State University, Fargo, ND 58108-6050
[*]Kow.chang@ndsu.edu**

Soybeans are subject to storage and shipping after harvest. During this period of time, the physical and biochemical characteristics of soybeans change to various degrees, depending upon the initial soybean moisture and storage/shipping conditions, including various humidities and temperatures. Inappropriate storage will lead to product quality defects as well as major economic losses. The changes in soybean characteristics may include darkening in surface color, increase in hydration time, reduction in solid and protein solubility, decrease in phytate content, isoflavone structural conversions, changes in protein structures, sugar pattern and lipid oxidation. The coagulant requirement per unit weight of soymilk for tofu making is reduced by improper storage. The yield and quality of soymilk and tofu are also negatively affected by severe storage conditions. Significant correlations were found among quality attributes, including color parameters (Hunter L, a, b, and ΔE), solid extractability (as expressed by soymilk solids content), soymilk pH and protein content, tofu yield, hardness and protein content. We have developed simple statistical equations and kinetic models, which can be used to monitor soybean quality and predict quality changes of soybeans during storage.

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Introduction

Soybeans are subject to storage and transportation before processing into various food products. They may be stored up to one year or longer after harvest in a wide variety of environmental circumstances. It is well known during shipping in a boat, the temperature of soybeans that are placed in close proximity of the engine compartment may be heated to higher than 50C. Furthermore, the long hot and humid summer months in Southeastern Asia, where soy foods are regularly consumed, cause soybean deterioration if they are not refrigerated. The quality of edible soybeans and the viability of soybean seeds decrease gradually with prolonged storage under certain conditions. The magnitude of the quality deterioration depends on storage conditions, including time, temperature, relative humidity (RH), and microbial contamination. Early studies on grain storage have shown that the initial moisture content is an important factor to influence quality stability during storage at 25C. Furthermore, it has been reported that temperature and relative humidity of the storage environment affect the chemical compositions of soybeans, but relative humidity is more important than temperature at high relative humidity range.

Soymilk and tofu are two major traditional soy foods (*1*). In the Western world, the consumption of soy foods and food containing soy has increased dramatically in the last decade, largely due to the approval of a heart health claim by the US FDA. Depending on storage conditions, the deterioration of soybeans may be reflected by several changes, including a decrease in hydration rate, an increase in the leakage of solids during soaking, an increase in the acidity of soybeans and darkening in bean color (*2*). The reported quality decreases in soymilk and tofu due to storage of soybeans include darkened soymilk/tofu color, low protein extractability (*2*), changes in tofu textural properties (*2*), and less uniformity in the microstructure of tofu (*3*). Tofu is a complex food system, and requires a series of processing steps from soybean raw materials. Tofu is considered as a protein gel and hence proteins in soymilk affect coagulation during tofu making. However, non-protein component, such as phytate has been reported to play an important role in the coagulation process (*4*). The coagulation of soymilk is the most critical step to influence yield and texture qualities, because it involves complex interactions among soy proteins, coagulants (usually Ca^{+2} and/or Mg^{+2}), and non-protein constituents such as phytate, lipids, and carbohydrate. The exact mechanisms of how soybean biochemical compositions change during storage concomitantly to affect tofu yield and quality are still not fully understood.

Most storage studies use certain accelerated-aging (adverse) conditions (*3*, *5*, *6*). It is commonly known that cold (refrigerated) environment is the best for long-term maintenance of grain quality. Storage under the conditions of 75% relative humidity at the ambient temperature is a critical factor for controlling storage fungi (*7*). However, discoloration and changes in biochemical constituents do occur during extended storage, even if visible growth of fungi has not occurred; and such changes would result in significant damages on soymilk and tofu quality. Soybeans after harvest are usually stored in uncontrolled ambient temperature and relative humidity conditions, which depend upon the regional environments. Despite the research reported by some researchers, comprehensive studies on maintaining food quality of soybeans stored over a wide range of temperature and humidity had not been carried out previously. Our research laboratory has focused on several objectives to the storage characteristics of food soybeans in the last fifteen years. Here I summarize our studies on the influence of the storage of soybean under various conditions on soybean biochemical components, color quality, coagulant requirement as related to soymilk/tofu making. This article also includes our recent studies on how soybean color changes and relates to the protein extractability, pH changes, and related to soymilk and tofu processing properties. Mathematical equations have been developed to describe these quality relationships as affected by storage temperature, humidity, and time of storage.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Influence of Physical Damage on Titratable Acidity and Other Quality Factors during Storage (*2*)

US graded soybeans (No. 1 and No. 2) allow 10-20% of splits. During harvesting and post-harvest handling, seed hull cracking may occur. It was of interest to understand how cracking and splitting affect food quality of soybeans stored under high temperature and humidity conditions. Soybeans were cracked to contain 10% and 20% of splits and seedcoat cracks, respectively, and were stored in 85% RH at 30 C for up to 60 days. Samples were retrieved at 15-day intervals and analyzed for determining chemical components, and soymilk and tofu processing quality. Titratable acidity of soybeans increased significantly from 0.9-1.0% to 1.12-1.23% during the 60 days of storage. Protein extractability in soymilk decreased significantly with storage time from 80% to 68% for the 20% split treatment. In general, the yield of tofu decreased significantly beyond 30 days of storage for all treatment groups and the control group which contained no damages or splits. The 20% split soybeans had a higher loss in tofu yield than 10% splits. Tofu could not be made from soybeans with 20% split and 60 days of storage. The color of tofu darkened, and the hardness, and the force to fracture tofu were significantly ($p<0.05$) decreased upon prolonged storage of soybeans. Total extractable soy proteins in soybeans were extracted from defatted soybean powder using 1% sodium dodecyl sulfate solution containing 50 mM mercaptoethanol as the reducing agent. Relative amount of glycinin (11S protein) in soybeans increased slightly as storage time increased for all treatments, but β-conglycinin (7S protein) did not change significantly with storage time. The results showed total proteins were not degraded. Therefore, the water insolubilization of proteins in soymilk making from adversely stored soybeans might be due to protein aggregations due to hydrophobic and disulfide linkages. Descriptive sensory evaluation of tofu made from soybeans stored for 45 days or longer at 85% RH and 30C showed significant off-flavor was produced under these adverse storage conditions.

Influence of Storage on Soybean Compositions, Color and Tofu Yield (*8*, *9*)

After harvest, soybeans are frequently stored for up to one year and sometimes longer than one year depending upon marketing. However, long-term storage studies of soybeans for more than one year are rarely conducted. It was of interest to study how long-term storage up to 18 months in the upper US Northern plains affects the quality of soybeans for making soy foods. Soybeans of the Proto variety (yellow with 44-45% protein content) were stored under four sets of conditions and analyzed for their changes in color, chemical compositions and tofu yield and textural characteristics. These four conditions included 1) adverse storage conditions (84% RH, 30C), 2) mild storage conditions (57% RH, 20C), 3) cold storage in a walk-in-cooler (86% RH and 3-4C), and 4) ambient conditions in a garage (humidity and temperature not controlled). The maximum duration was nine months for the adverse storage, and 18 months for the other three conditions. Soybean color significantly changed over time during adverse storage (Table I). Generally, the whiteness of soybeans (Hunter L* values) decreased along with storage time from the lightness value of 51 to 39 over the nine months of the adverse storage, indicating that soybean became darker. Redness values (Hunter a* values) increased up to a maximum at five months of storage. However, yellowness (Hunter b* values) decreased gradually over the time of storage.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Table I. Changes of the Surface Color of Soybean of the Yellow Proto Variety during Adverse Storage for up to Nine Months*

84% RH, 30 C Month (m)	*Whiteness, L**	*Redness, a**	*Yellowness, b**
Control, 0 m	51	4.1	16
1 m	50	4.2	15
2 m	50	4.6	15
3 m	49	4.8	15
4 m	48	5.2	15
5 m	45	5.5	13
6 m	43	4.8	11
7 m	45	5.1	12
8 m	42	4.3	11
9 m	39	3.9	9

Soybeans in the adverse environment absorbed significant amount of moisture over time, the moisture content increased from the original 5.4 % to about 18 % at the end of nine months. Soybeans at the bottom of the containers under the adverse storage conditions began molding after 3 months and generated off-flavor. Those molded soybeans were carefully picked out and discarded to avoid the influence of molds on tofu and chemical compositions. The lipid, proteins, ash and total carbohydrate content in the beans did not change significantly over the entire duration of storage. However, sugar content decreased significantly from about 12 to 6% under the adverse storage conditions.

Soybeans stored under the adverse conditions showed significant decrease the recovery of protein in the extracted soymilk (Figure 1) and significant decreases in tofu yields from 512 g to 71 g per 100 g soybean (Table II) after 7 months of storage, whereas textural quality decreased even only after 2-3 months of storage(Table II). No tofu gel could be formed after 7 months of storage under the adverse conditions. The tofu hardness increased significantly with soybean storage time. Beyond three months of storage, the tofu yield decreased significantly with a concomitant increase in tofu hardness up to more than 5 kg force by the 6th month of storage. A high negative correlation existed between tofu yield and tofu hardness. The coarse and firm texture resulted from the adverse storage is not desirable for soft-tofu type of the end products. Soybeans stored under mild, cold, and uncontrolled ambient conditions showed no significant changes in their color or compositions except for moisture, and maintained good tofu making quality for up to 18 months.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

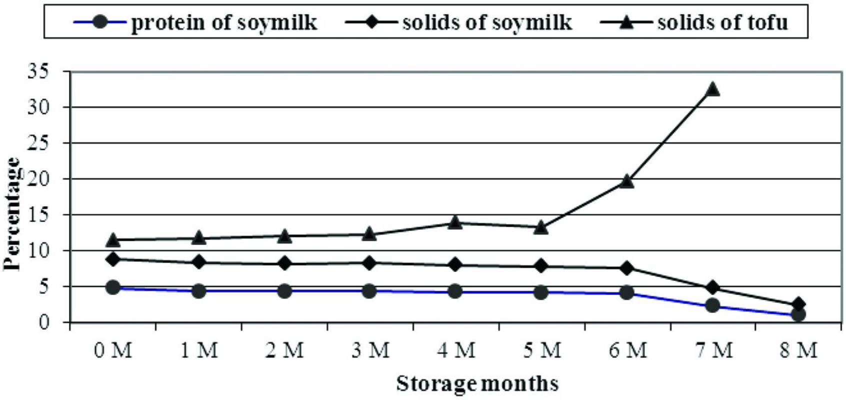


Figure 1. Effect of adverse storage conditions of 84% RH and 30C on the protein, solid recoveries in soymilk and tofu solid prepared from Proto soybeans. SOURCE: Reference (9).

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Table II. Yield and Textural Properties of Tofu Made from Soybeans Stored in 84% RH and 30 C[*]

Month	*Yield (g/100 g bean)*	*Textural properties*	
		Brittleness (g)	*Hardness (g)*
0	511.5 ± 4.9[a-f]a	1020 ± 56[a-f]bc	2090 ± 42[a-f]f
1	504.5 ± 2.1[a-f]a	1033 ± 81[a-f]b	2182 ± 12[a-f]f
2	481.0 ± 2.8[a-f]a	923 ± 25[a-f]cd	2465 ± 21[a-f]e
3	421.0 ± 5.7[a-f]b	873 ± 11[a-f]d	2704 ± 107[a-f]d
4	388.5 ± 0.7[a-f]bc	920 ± 57[a-f]cd	3080 ± 113[a-f]c
5	362.5 ± 43.1[a-f]c	945 ± 14[a-f]bcd	3278 ± 79[a-f]b
6	232.5 ± 3.5[a-f]d	>5000[a-f]a	>5000[a-f]a
7	71.0 ± 1.4[a-f]e	>5000[a-f]a	>5000[a-f]a
8	No tofu was made	NA	NA
9	No tofu was made	NA	NA

[*] Data are expressed as means ± s.d. Data of yield are means of 2 replicates on a wet weight basis (5.4 % moisture). [a-f] means with different letters within the same column differ at ($p<0.05$). NA: Not available. SOURCE: Reference (*9*).

Influence of Storage on Soybean Phytate Content and Its Relationships with Tofu Yield and Quality

Since phytate was previously reported to affect tofu coagulation and textural qualities (*4*), we carried out experiments to attempt to understand the effect of phytate changes (decreases or increases) on the coagulation, yield, and texture of tofu (*9*). In the experiments described in the above section on the storage of Proto soybeans under the four sets of conditions, phytate content in soybean stored under the adverse conditions showed a linear decrease with storage time. At the end of three months of adverse storage, phytate was decreased from 1.33% to 1.16%. At the end of nine-month storage, phytate content declined to 0.87 %, which was a 35% reduction from the initial amount. The titratable acidity of soybeans increased linearly from 0.73% to 2.03% (increased by 178%) and pH of the soymilk decreased from 6.6 to 5.5 after nine months of storage under the adverse conditions. The phytate content of soybean stored under the three other conditions did not change after 12 months and decreased slightly thereafter (8 % to 13% by the18th months). Titratable acidity also increased slightly (approximately 12%) in the three other storage conditions by the end of 18 moths. The increase in soybean titratable acidity could be partly attributed to an increase in free fatty acids and phosphoric acid due to the degradation of lipids and phytate by enzymes lipase and phytase, respectively.

In order to study how decreases in phytate during storage affect the tofu yield and quality of the Proto soybeans, the phytate of the soymilk made from the fresh soybean (control soybeans stored in a freezer at -20C) was selectively reduced by 11.3% and 17.5% by phytase, while other components remained unchanged. These phytate reduction levels are equivalent to the phytate reductions in soybeans stored for 2 to 4 months under the adverse conditions. The tofu yield of the phytase catalyzed phytate-reduced soymilk increased significantly, but the texture became softer. These effects were opposite to the tofu yield decreases and hardness increases observed under the adverse storage conditions.

Furthermore, we conducted an experiment to increase the phytate content by adding the lost phytate back into the soymilk that was made from adversely stored soybeans up to four months to compensate for the reductions in phytate. We found that the addition of external phytate did not affect yield at 2 months of storage, but even decreased the yield of tofu at 3 and 4 months of storage under adverse conditions. There was a tendency to decrease pH when phytate was added into the soymilk. Addition of phytate also resulted in the lowering of the force to fracture the tofu. Furthermore, we added up to 50% of the phytate to the soymilk made from fresh soybeans to understand if phytate would change the yield and texture of tofu. The results showed that adding more phytate did not have any effect of either tofu yield or textural quality. Therefore, phytate degradation during adverse storage played a minor, indirect role by contributing acidity to the deterioration of soybeans for tofu making.

The low yield of tofu made from soybeans stored under the adverse conditions was not due to phytate content, but primarily due to two factors: the low soymilk protein recovered and dramatic increase in titratable acidity. The solids content in soymilk remained above 8% for 4 months, then was reduced to about 7.5% in 6 months and continually decreased to less than 2.4% in 8 months. The protein content in the wet soymilk showed a similar decreasing trend with the solids content of soymilk; it reduced from 4.9% to 4.4% in the first month then remained above 4% until 6 months. By the end of the 8th month, the protein content in the wet soymilk was only about 1%.

Compared to the adverse conditions of 84% RH, 30C, the solids contents in soymilk for the other three conditions did not change significantly, ranging from 8.40-8.94 %, over the storage time. Similarly, protein content in wet soymilk remained at above 4.5% for these three conditions over the storage time. Solids contents in tofu made from stored soybeans under these three conditions ranged from 11.2% to 11.8%, and did not show substantial changes over time of 18 months. The yield and textural properties of the tofu made from the soybean stored in the other three mild, cold and garage conditions for up to 18 months did not change substantially.

The high titratable acidity after adverse storage plays an important role in tofu yield decrease, because acid could act as a coagulant to cause pre-gelation prior to the addition of coagulant or to accelerate coagulation after the addition of coagulant. Besides the protein factor, the decreases in tofu yield were due mainly to the loss of the water that was pressed out that should have been imbibed by the proteins inside the curd. The sharp increase in solids content in tofu particularly beyond 5 months of adverse storage indicated the inability of the curd matrix to imbibe water resulting in a significant decrease in moisture and yield. The lower the moisture content, the harder the tofu texture produced.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Influence of Storage on β-Conglycinin and Glycinin Protein Structures

Since gelation of soymilk in the tofu making process depend on the behavior of the two major storage proteins, β-conglycinin and glycinin. For understanding how adverse storage affects the protein structures (not only protein solubility and content), these two major storage proteins were isolated and purified to electrophoretic homogeneity from the soybeans stored under the four storage conditions including adverse conditions (84% RH, 30C), mild conditions (57% RH, 20C), cold conditions (4C), and uncontrolled ambient conditions in a car garage (*10*, *11*). The storage time was 9 months for the adverse conditions and 18 months for the other three conditions. The secondary and tertiary structures of these proteins were analyzed to determine their potential impact on the behavior of curd coagulation and yield. β-Conglycinin and glycinin were purified with a combination of solvent fractionation and chromatographic methods, and characterized with respect to its molecular properties. The extractability of β-conglycinin from soybeans stored in the adverse conditions decreased significantly after 2 months and dramatically after 6 months of storage. Beyond six months, most β-conglycinin became unextractable due to structural changes **(**Table III), presumably protein aggregations resulting in the loss of water solubility. In the adverse conditions, β-conglycinin showed insignificant changes in total sugar content (glycosylation), surface hydrophobicity **(**Table III**),** free SH and SS bonds, and amino acid composition within 6 months. However, a significant decrease in surface hydrophobicity and a significant increase in total free SH and total SH including SS content after 6 months. The analysis of secondary structures showed a significant increase in α-helix content from, but a significant decrease in β-sheet content after 3 months (Table IV). For the other three storage conditions, no significant changes occurred to the structures of β-conglycinin when compared to the control. The molecular mass of β-conglycinin remained in the range of 199~212 kDa for all conditions during the entire storage periods. The denaturation temperature analyzed by differential scanning calorimetry did not show any changes during storage of all four sets of the storage conditions with the peak temperatures for β-conglycinin and glycinin at approximately 79 and 98C, respectively.

The extractability of glycinin also decreased with the increase of adverse storage time. The crude yield of glycinin was almost zero after 8 months of storage. The structures of purified glycinin from soybean stored in the adverse conditions showed changes after 3 months with an increase in glycosylation and α-helical secondary structures (14.9 to 16.6% by the 8th month), and decreases in β-pleated sheet (36.7 to 33.1% by the 8th month) (Table V), and hyrophobicity. However, the glycinin from soybeans stored under other three conditions had no significant changes after 18 months when compared to that isolated and purified from the control soybeans. Glycinin from soybeans in the adverse conditions was associated with a significant amount of glycosylation and showed decreases in hydrophobic interactions after 3 months of storage. The interchange reactions between SH and S-S within glycinin molecules increased due to a decrease in total free SH content but an increase in SS content. On the contrary, the hydrogen bonding within the glycinin molecule might decrease because an increase in α-helix structure but a decrease in the β-sheet structure. The molecular mass of purified glycinin remained in the range of 313~340 kDa after storage for all conditions. The changes in glycinin structure due to the adverse storage may negatively affect soy product quality, especially for tofu that forms gel matrix from glycinin. Stable structures found in β-conglycinin and glycinin, which were purified from the three other conditions for 18 months are consistent with the fact that soybeans maintained their good qualities for making tofu under these storage conditions.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Table III. The Isolation Yield of Crude β-Conglycinin and Total Sugar Content and Surface Hydrophobicity of β-Conglycinin Purified from Soybeans under Various Conditions[#]

Storage Conditions Month (m)	*Crude Protein Yield*[*] *(%)*	*Total Sugar Content*[♣] *(%)*	*Surface Hydrophobicity (cps x* 10^6*)*
Control	12.59 ± 0.38$^{a\text{-}g}$a	4.27 ± 0.03$^{a\text{-}g}$c	3.41 ± 0.29$^{a\text{-}g}$bc
84%RH, 1 m	11.61 ± 0.12$^{a\text{-}g}$b	4.73 ± 0.06$^{a\text{-}g}$ab	3.14 ± 0.05$^{a\text{-}g}$cd
84%RH, 2 m	9.28 ± 0.49$^{a\text{-}g}$c	4.65 ± 0.03$^{a\text{-}g}$b	3.04 ± 0.13$^{a\text{-}g}$de
84%RH, 3 m	5.74 ± 0.11$^{a\text{-}g}$d	4.58 ± 0.09$^{a\text{-}g}$b	3.71 ± 0.09$^{a\text{-}g}$b
84%RH, 4 m	5.54 ± 0.41$^{a\text{-}g}$de	4.67 ± 0.19$^{a\text{-}g}$b	2.74 ± 0.14$^{a\text{-}g}$ef
84%RH, 5 m	4.97 ± 0.57$^{a\text{-}g}$de	4.79 ± 0.11$^{a\text{-}g}$ab	2.86 ± 0.17$^{a\text{-}g}$def
84%RH, 6 m	5.12 ± 0.38$^{a\text{-}g}$de	4.83 ± 0.12$^{a\text{-}g}$a	3.01 ± 0.07$^{a\text{-}g}$de
84%RH, 7 m	1.63 ± 0.21$^{a\text{-}g}$f	4.89 ± 0.04$^{a\text{-}g}$a	3.03 ± 0.04$^{a\text{-}g}$de
84%RH, 8 m	1.45 ± 0.32$^{a\text{-}g}$f	4.77 ± 0.12$^{a\text{-}g}$a	2.54 ± 0.05$^{a\text{-}g}$f
84%RH, 9 m	0.19 ± 0.11$^{a\text{-}g}$g	NA[♠]	2.12 ± 0.26$^{a\text{-}g}$g
57%RH,18 m	12.15 ± 0.37 $^{a\text{-}g}$a	4.27 ± 0.11$^{a\text{-}g}$c	3.39 ± 0.32$^{a\text{-}g}$bc
4 °C, 18 m	12.51 ± 0.22 $^{a\text{-}g}$a	4.45 ± 0.16$^{a\text{-}g}$bc	4.09 ± 0.44$^{a\text{-}g}$a
Ambient, 18 m	12.23 ± 0.15 $^{a\text{-}g}$a	4.30 ± 0.07$^{a\text{-}g}$c	3.40 ± 0.26$^{a\text{-}g}$bc

[#] Data was expressed as mean ± standard deviation, and was mean of three replicates. [*] The crude β-conglycinin yield was calculated based on the total protein of dried soybeans. [♣] The sugar content was based on dried purified β-conglycinin. [♠] Sample was not available for determination. $^{a\text{-}g}$ means with different superscripts within the same column are significantly ($p<0.05$) different. SOURCE: Reference (*11*).

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Table IV. Secondary Structure of β-Conglycinin Purified from Soybeans Stored Under Various Conditions*

	Secondary Structure (%)				
Storage Condition	*α-helix*	*β-sheet*	*β-turn*	*Unordered*	*Total*
Control, 0 month	13.27^{a-c}b	37.89^{a-c}a	24.69	23.02	98.87
84%RH, 30 C, 3 m	15.37^{a-c}a	34.47^{a-c}c	24.91	24.23	98.98
84%RH, 30 C, 6 m	15.08^{a-c}a	35.60^{a-c}b	24.79	23.72	99.19
84%RH, 30 C, 8 m	15.08^{a-c}a	34.38^{a-c}c	24.75	24.12	98.33
57%RH, 20 C, 18 m	12.92^{a-c}b	35.81^{a-c}b	24.24	22.55	95.52
Cold, 4 C, 18 m	12.53^{a-c}bc	37.09^{a-c}a	23.85	22.11	95.58
Ambient Temp. 18 m	12.32^{a-c}c	38.44^{a-c}a	24.23	22.60	97.59

* Data are expressed as percentage and are the mean of two replicates. $^{a-c}$ means with different superscripts within the same column are significantly ($p<0.05$) different. SOURCE: Reference (*11*)

Table V. Secondary Structure of Glycinin Purified from Soybeans Stored under Various Conditions*

	Secondary Structure (%)				
Storage Condition	*α-helix*	*β-sheet*	*β-turn*	*Un-ordered*	*Total*
Control, 0 month	14.88^{a-d}c	36.66^{a-d}b	24.91^{a-d}b	22.66^{a-d}c	99.11
84% RH, 30C, 3 m	15.27^{a-d}bc	35.96^{a-d}bc	24.20^{a-d}b	21.16^{a-d}d	96.59
84% RH, 30C, 6 m	16.07^{a-d}ab	35.18^{a-d}c	24.05^{a-d}b	21.30^{a-d}d	96.60
84% RH, 30C, 8 m	16.60^{a-d}a	33.10^{a-d}d	24.92^{a-d}b	24.60^{a-d}b	99.22
57% RH, 20C, 18 m	15.43^{a-d}b	33.79^{a-d}d	24.72^{a-d}b	25.62^{a-d}a	99.56
Cold, 4 C, 18 m	15.63^{a-d}b	36.32^{a-d}b	26.38^{a-d}a	24.63^{a-d}b	102.96
Ambient Temp. 18 m	14.97^{a-d}c	37.64^{a-d}a	24.32^{a-d}b	22.91^{a-d}c	99.84

* Data are expressed as percentage and are the mean of two replicates. $^{a-d}$ Means with different letters in the same column differ ($p<0.05$). SOURCE: Reference (*10*).

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

In further studies (*12*, *13*), we fixed the protein content in the soymilk and found that tofu quality decreased as storage conditions became more severe. The results further proved that protein structural changes during adverse storage, including increases in α-helix, disulfide linkages and glycosylations, decrease in β-pleated sheets and hydrophobicity contributed to the loss in the ability of the soybean storage proteins to form a finely cross-linked gel network of tofu curd, thereby producing coarse tofu and low tofu yield.

Influence of Storage on Isoflavones (*14*)

Soybeans and soy foods are the major food sources containing nutritionally relevant amounts of isoflavones. There are 12 forms of isoflavones in soybeans that are aglycones, and glucosides, acetyl glucosides and malonyl glucosides of aglycones: genistein, daidzin and glycitein. Isoflavones have been reported by many researchers to be the bioactive compounds in soy that contribute to the reduction of several types of chronic disease in East Asian countries. Isoflavones of different types also have been shown to have different bitterness. The enzyme β-glucosidase can hydrolyze glucosides of isoflavones to aglycones. We have characterized the conversion of isoflavones in the soybeans stored under the previously described four storage conditions. Significant interconversions ($p<0.001$) between aglycones and β-glucosides of isoflavones occurred in soybeans stored under the adverse condition (84% RH and 30C). Soybeans contained 99% of the total isoflavones in the forms of β-glucosides and malonylglucosides before storage, but contained only 3% after 9 months of storage under the adverse storage conditions. However, the percentage of aglycones in the total isoflavones increased from 1% to 97% in 9 months. Therefore, during adverse storage of soybeans, glucosides are almost all converted to aglycones. Under the mild cold conditions (57% RH, 20C), the contents of β-glucoside forms in the total isoflavones increased from 12% to 31% in 18 months, but the contents of malonylglucosides decreased from 87% to 66%. Under the cold condition (4C), isoflavone distributions had no significant ($p>0.05$) changes during storage. Under the uncontrolled ambient conditions, the changes between β-glucoside forms and malonylglucosides were similar to that occurred in soybeans stored under mild storage conditions. The contents of β-glucosides in the total isoflavones increased from 12% to 22% in 18 months, but malonyl forms decreased from 87% to 76%. Studies have shown that aglycones are more bioavailable than the glucosides. However, the full significance of the changes of isoflavones to the food quality and physiological impact remains to be understood.

Influence of Storage on Coagulant Requirements and Mixing Time during Tofu Making (*15–17*)

The proper use of coagulant concentration is very important in tofu making since coagulation (gelation) of proteins affects textural quality, and also taste of the products. Too high or too low concentrations of the coagulants will result in poor textural quality. In addition, how much the soymilk is mixed is very important to affect the type of firmness of tofu gel. Each type of tofu requires a combination of proper coagulant concentrations and mixing time. We have developed a rapid method for measuring the optimal coagulant concentrations (*16*). Furthermore, we have studied how storage can affect optimal concentration requirements of the soymilk in making soft tofu (*15*). In this storage study, soybeans were adjusted to water activity (Aw) from 0.60 to 0.81, sealed in containers, and stored in a 30C incubator for up to three months. Soybeans were retrieved at one-month interval and processed into soymilk with 10.5 Brix solids content by adjusting water-to-bean ratio. The optimal coagulation concentration for making filled soft tofu was determined using a rapid titration method which was developed in our laboratory (*16*). Other soymilk characteristics, including protein, 11S/7S ratio, titratable acidity, pH and color, were also determined. Filled tofu was prepared from soymilk using either $MgCl_2$ or $CaCl_2$ as the coagulant. The results showed optimal coagulant concentrations decreased with the increases in Aw of soybeans, and also decreased with the increase in storage time (Table VI). The decreases in optimal coagulant concentration were associated with the increases in soymilk titratable acidity and the decreases in soymilk pH. The instrumental tofu textural properties such as breaking strength and Young's modulus decreased slightly with the storage time. In addition, soymilk and tofu's lightness and yellowness decreased, whereas their redness increased. These color changes were consistent with our previous findings on the soybeans stored under adverse conditions. In another study on the determination of the coagulant requirements of 33 soybeans, in which several soybeans samples were stored at the room temperature for several years, we found a decrease in coagulant requirements over the time of storage (*16*).

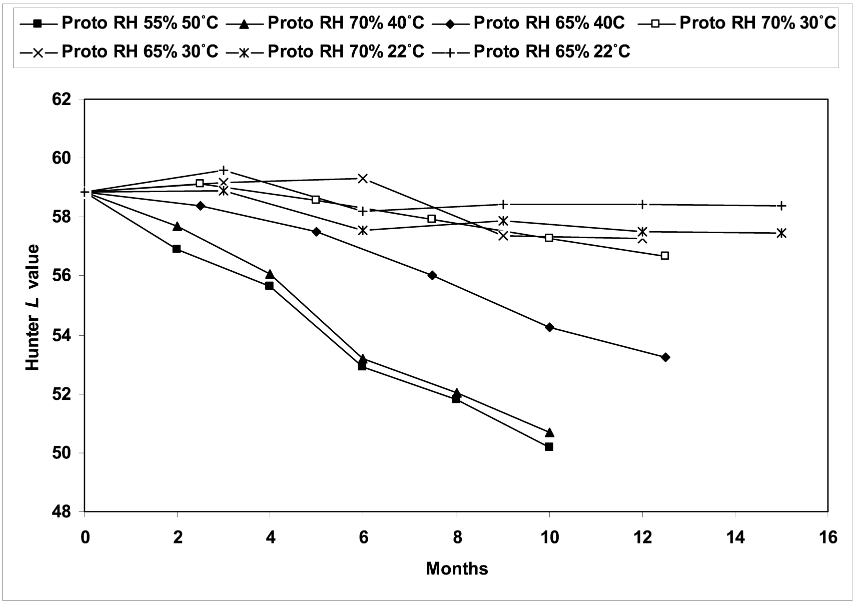


Figure 2. Hunter L (Lightness) changes of 2001-harvested Proto soybeans during storage. Each point represents average of five measurements. SOURCE: Reference (17).

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Table VI. Effect of Aw of Soybeans and Storage Time at 30 C on Optimal Coagulant Concentration (OCC) of $CaCl_2$ used for Making Filled Tofu. Conditions of 84% RH and 30 C

Aw	*1 Month, mM $CaCl_2$*	*2 Month, mM $CaCl_2$*	*3 Months, mM $CaCl_2$*
0.60	12.2	11.5	10.8
0.63	11.9	10.9	9.8
0.66	11.4	11.1	9.8
0.69	11.6	10.2	8.3
0.71	10.9	9.5	8.6
0.74	10.8	9.7	8.7
0.76	10.6	9.3	8.4
0.78	9.9	8.6	8.1
0.80	9.8	8.4	7.6
0.81	9.0	7.9	7.7

In another study (*17*) we fixed the propeller blade size, mixing speed and coagulation concentration, we found that optimal mixing time for producing the best soft tofu from the stored beans decreased with the adverse storage duration**.** Figure 2 illustrates the decrease in optimal mixing time from about 10 sec to 6.5 sec for Proto soybeans stored at 70%RH and 40C.

Predicting Soymilk and Tofu Making Quality from Surface Color and Soybean Storage Conditions (*17*, *18*)

Developing effective and rapid methods for monitoring and predicting soybean quality for making soy foods is desirable to the soybean trade and the manufacturing industries. One way is to develop the kinetic and mathematical equations using simple physical and chemical measurements. To achieve this goal, storage of soybeans under a wide range of relative humidity and temperature conditions were carried out and soybean quality as well as soymilk and tofu-making properties were analyzed. Soybeans of three different genotypes (Proto, Vinton 81 and a lipoxygenases-null soybean IA2032) were stored in varying conditions: temperature ranging from 4 to 50C, relative humidity from 55 to 80%, initial moisture content from 6 to14%, and storage time up to 15 months depending upon storage conditions. The effects of different storage conditions on soybean color, solids and protein extractability, soymilk pH, tofu yield, tofu solids and protein contents, tofu color and texture were investigated. While no significant changes occurred for the soybeans stored at 4C, the soybeans stored at high temperatures (30-50C) exhibited significant quality losses ($P < 0.05$). The degradation of soybean lightness (Hunter L^*) (Figure 3), color difference (ΔE) and solid extractability exhibited a linear relationship with time. Soak

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

weight decreased from soybeans stored at high temperature and relative humidity, while increased when stored at mild storage conditions. Figure 4 illustrates the decreases in protein extractability in soymilk for soybeans stored under a wide range of relative humidity and temperature. Several combinations of the storage conditions at temperatures exceeding 30C produced drastic losses in tofu yields. Storage also affected tofu making process by reducing optimum mixing time to produce the highest tofu yield. Varietal differences in soybean storability were observed with Vinton deteriorated faster than IA2032 (lipoxgenases-null) and Proto since Vinton contained lipoxygenases and had higher lipid content than Proto (*19*). The results provided useful information for soybean processing industry to store soybeans using the optimal storage conditions and to estimate soybean quality after storage.

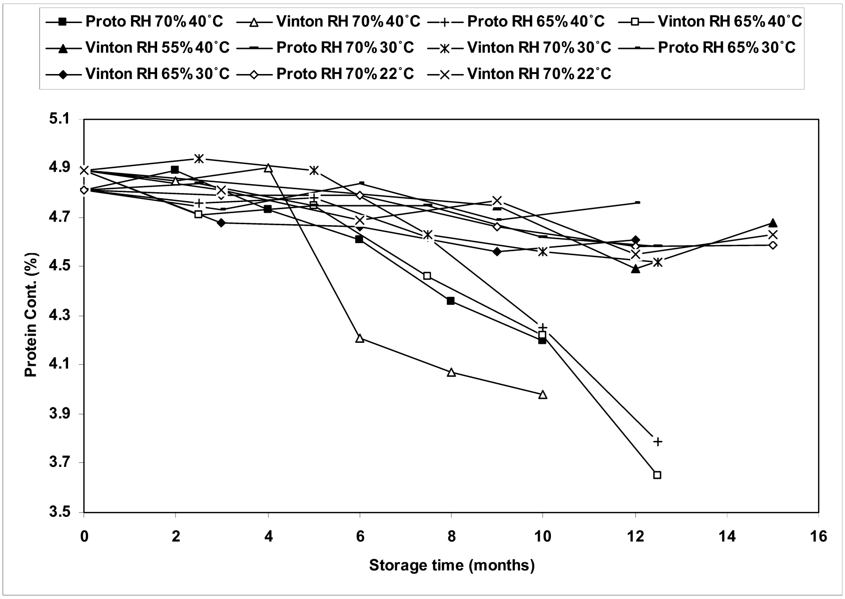


Figure 3. Decreases of protein content in soymilk made with 2001-harvested Proto and Vinton soybeans after storage at different conditions. Each point represents average of two measurements. The average standard deviation =0.01%. SOURCE: Reference (17).

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

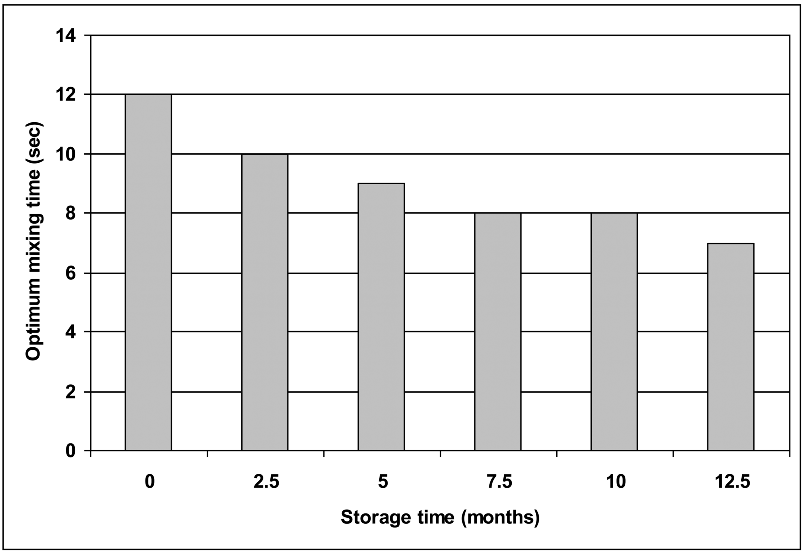


Figure 4. Decreases of optimum mixing time (t_m) in the tofu making process with 2001-harvested Proto after storage at RH 70% and 40 °C. SOURCE: Reference (17).

Based on the results of the above comprehensive storage and analysis, mathematical equations and kinetic models (*18*) were developed to describe the relationships between the changes of soybean quality attributes and soymilk and tofu making quality (*17*). Significant correlations ($P<0.05$) were found among most of quality attributes, including color parameters (Hunter L^*, a^*, b^*, and ΔE), solid extractability (as expressed by soymilk solids content), soymilk pH and protein content, tofu yield, hardness and protein content. Regressed linear equations were developed between color indices (L^*/L_0^*, ΔE) and soymilk/tofu making properties (Table VII). Mathematical equations have been developed to relate soybean color indices (L^*/L_0^*, ΔE) and storage conditions, using experimental values of the four variables of initial moisture content (*MC:6-13%*), relative humidity (*RH:55-80%*), temperature (*T: 4-50C*) and duration (t: *up to 12 months of storage*).

Kinetics of the changes in soybean color and extractability during storage at 70% *RH* and 22–40 °C were investigated. The kinetics was well described by zero-order kinetics. The Arrhenius equation adequately described the temperature dependence of the reaction rate constants for all parameters, from which the activation energies and rate constant were obtained. The equations developed in this study provided simple methods to monitor soybean quality and predict quality changes of soybeans during storage under various conditions.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Table VII. Linear Model Equations between Soymilk/Tofu Making Properties and Soybean Color Indices[*]

y	*x*	*Model equation*	*r*	*No. of Measurements*
		a. Proto variety		
Y/Y_0	L/L_0	y = 2.948x - 1.993	0.68	25
Y/Y_0	ΔE	y = -0.057x + 1.010	-0.77	25
S/S_0	L/L_0	y = 1.959x - 0.973	0.95	30
S/S_0	ΔE	y = -0.032x + 1.013	-0.96	30
P/P_0	L/L_0	y = 0.981x + 0.017	0.72	30
		b. Vinton 81variety		
Y/Y_0	L/L_0	y = 2.949x - 1.997	0.73	24
Y/Y_0	ΔE	y = -22.72x + 495.7	-0.63	24
S/S_0	L/L_0	y = 1.815x - 0.850	0.95	30
S/S_0	ΔE	y = -0.031x + 0.997	-0.95	30
P/P_0	L/L_0	y = 1.495x - 0.511	0.89	30
P/P_0	ΔE	y = -0.026x + 1.014	-0.90	30
		c. Combination of Proto, Vinton 81 and IA2032		
Y/Y_0	L/L_0	y = 2.789x - 1.830	0.70	88
Y/Y_0	ΔE	y = -0.046x + 0.998	-0.68	88
S/S_0	L/L_0	y = 1.890x - 0.914	0.94	60
S/S_0	ΔE	y = -0.031x + 1.006	-0.95	60
P/P_0	L/L_0	y = 1.229x - 0.239	0.78	60
pH/pH_0	L/L_0	y = 0.636x + 0.350	0.93	38
pH/pH_0	ΔE	y = -0.011x + 0.996	-0.93	38

[*] L, ΔE, S, P, Y represent soybean lightness, color difference, soymilk solids content, protein content, and tofu yield, respectively; L_0, pH_0, S_0, Y_0 and P_0 were the initial values. Reference (*18*).

Conclusion

The color and biochemistry of the storage-induced changes in soybeans as related to soymilk and tofu were systematically investigated in a series of research projects in our laboratories. Soybeans stored under the adverse conditions (84% RH and 30C) deteriorated significantly after 2 months; and the deteriorations were reflected in the generation of off-flavor, decreases in tofu yield, and coarser and harder of tofu texture. Soybeans stored in the mild conditions such as 57% RH and 20C could remain their qualities for tofu making as good as those stored in the cold conditions (4C) for up to 18 months. Soybeans stored in the uncontrolled temperature garage conditions in the US Northern Plain region for up to 18 months also could maintain their qualities for tofu making without substantial differences as compared to those stored in the cold conditions. The coagulant concentration of the soymilk decreased over time and water activity of the storage. We have developed mathematical equations for predicting soybean quality for soymilk and tofu making. Overall, the best storage conditions for storing soybeans are below 22C, and 55-60% RH, which corresponds to initial moisture content of 8 to 10% in the soybeans, respectively. Under these conditions, soybeans can be stored for more than one year without significant changes of soymilk and tofu quality. The information provided is useful for the soybean and food industries to manage the storage practice to maximize the quality of the soymilk and tofu products. The study is also important to alert the tofu makers that coagulant concentrations must be modified accordingly for stored beans during tofu making if the optimal concentration is significantly affected by storage.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

Acknowledgments

ND AES #2707 and 1905, ND Soybean Council, USDA-CSREES NRI CGP #2001 01583 and US-NSF-EPSCoR Doctoral Fellowship Project #4414, provided funding for this project. The technical support of Dr. H. J. Hou, Dr. Z. Liu, and Dr. F. B. Kong are appreciated.

References

1. Chang, S. K. C. Soymilk and Tofu Manufacturing. In *Handbook of Food Product Manufacturing*; Hui, Y. H., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2007; Chapter 95, pp 1063−1089.
2. Hou, H. J.; Chang, K. C. Yield and quality of soft tofu as affected by soybean physical damage and storage. *J. Agric. Food Chem.* **1998**, *46*, 4798–4805.
3. Thomas, R.; deMan, J. M.; deMan, L. Soymilk and tofu properties as influenced by soybean storage conditions. *J. Am. Oil Chem. Soc.* **1989**, *66*, 777–782.
4. Saio, K.; Koyama, E.; Yamazaki, S.; Watanabe, T. Protein-calcium-phytic acid relationships in soybean Part III. Effect of phytic acid on coagulative reaction in tofu-making. *Agric. Biol. Chem.* **1969**, *33*, 36–42.
5. Saio, K.; Nikkuni, I.; Ando, Y.; Otsuru, M.; Terauchi, Y.; Kito, M. Soybean quality changes during model storage studies. *Cereal Chem.* **1980**, *57*, 77–821980.
6. Lambrecht, H. S.; Nielsen, S. S.; Liska, B. J.; Nielsen, N. C. Effect of soybean storage on tofu and soymilk production. *J. Food Qual.* **1996**, *19*, 189–202.
7. Christensen, C. M.; Kaufmann, H. H. *Grain Storage: The Role of Fungi on Quality Loss*; University of Minnesota Press: Minneaplis, MN, 1969.
8. Hou, H. J.; Chang, K. C. Storage affects color and chemical composition and tofu making quality. *J. Food Process. Preserv.* **2004**, *28*, 473–488.

Downloaded by PENNSYLVANIA STATE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch008

9. Hou, H. J.; Chang, K. C. Yield and textural properties of soft tofu as affected by changes of phytate content during soybean storage. *J. Food Sci.* **2003**, *68*, 1185–1191.
10. Hou, H. J.; Chang, K. C. Structural characteristics of purified glycinin from soybeans stored under various conditions. *J. Agric. Food Chem.* **2004**, *52*, 3792–3800.
11. Hou, H. J.; Chang, K. C. Structural characteristics of purified beta-conglycinin from soybeans stored under various conditions. *J. Agric. Food Chem.* **2005**, *52*, 7931–7937.
12. Kong, F. B.; Chang, S. K. C. Changes in Protein Characteristics during Soybean Storage under Selected Adverse Conditions. 68th Annual Meetings of the Institute of Food Technologists, Chicago, IL, July 28−August 1, 2007.
13. Kong, F. B. Changes in Soybean Quality during Storage as Related to Soymilk and Tofu Making. M.S. Thesis, North Dakota State University, Fargo, ND, 2006.
14. Hou, H. J.; Chang, K. C. Interconversion of isoflavones in soybeans as affected by storage. *J. Food Sci.* **2002**, *67*, 2083–2089.
15. Liu, Z. S.; Chang, S. K. C. Optimal coagulant concentration, soymilk and tofu quality as affected by a short-term model storage of Proto soybeans. *J. Food Process. Preserv.* **2008**, *32*, 39–59.
16. Liu, Z. S.; Chang, K. C. Development of a titration method for predicting the optimal concentrations of coagulants for making filled tofu. *J. Agric. Food Chem.* **2003**, *51*, 5214–5221.
17. Kong, F. B.; Chang, S. K. C.; Liu, Z.; Wilson, L. A. Changes of soybean quality during storage as related to soymilk and tofu making. *J. Food Sci.* **2008**, *73*, S134–144.
18. Kong, F. B.; Chang, S. K. C. Statistical and kinetic studies of soybean quality during storage as related to soymilk and tofu making. *J. Food Sci.* **2009**, *74*, S81–89.
19. Yuan, S. H.; Chang, S. K. C. Selected beany odors in soymilk as affected by chemical composition, and lipoxygenases in five soybean materials. *J. Agric. Food Chem.* **2007**, *55*, 426–427.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Chapter 9

Chemistry and Biological Properties of Soybean Peptides and Proteins

Elvira Gonzalez de Mejia* **and Vermont P. Dia**

Department of Food Science and Human Nutrition,
University of Illinois at Urbana-Champaign, Urbana, IL 61801
***edemejia@uiuc.edu**

Soybean, an important source of food proteins, has received increasing interest from the public because of its reported health benefits. These health benefits are attributed to its components which include isoflavones, saponins, proteins and peptides. Lunasin, Bowman-Birk inhibitor, Kunitz trypsin inhibitor and β-conglycinin are some of the biologically active peptides and proteins found in soybean. This chapter provides a comprehensive review on the biological properties and the recently used techniques in the analysis and characterization of soy bioactive peptides. Also, the basic chemistry of each of the bioactive peptides is discussed. In sum, bioactive peptides can be accurately identified and quantified using different techniques and conditions which are important in order to accurately characterize their individual biological activities.

Food proteins are important nutrients as they provide essential amino acids for proper maintenance of life. In food processing, they provide essential functionality in food systems as they are involved in the formation of gels, the structure of dough, the texture of food products, the stability of emulsions as well as water binding capacity. In the past 20 to 30 years, another aspect of food proteins has been recognized; in particular, the capability of protein derived peptides and naturally occurring peptides which can exert certain biological activities. In a review by Wang and de Mejia (*1*), it is shown that these biologically active peptides, whether derived by hydrolysis and/or fermentation or naturally present, can exhibit functional health benefits including immunomodulation,

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

cancer prevention, hypotensive and antioxidant properties, hypocholesterolemic and anti-obesity characteristics. One important food that contains biologically active peptides is soybean.

The reported health benefits of soybean are attributed to its content of isoflavones, saponins, peptides and proteins (*2*). Soybean contains an appreciable amount of proteins, in dry basis, accounting for as much as 48%. The majority of these proteins are associated to soybean storage proteins namely glycinin and β-conglycinin. Peptides derived from these proteins, either by enzymatic hydrolysis or by fermentation, exert specific biological activities (*2*, *3*). For example, enzymatic hydrolysis of glycinin and β-conglycinin results in the production of bioactive peptides with anti-hypertensive (*3*, *4*), hypocholesterolemic (*5*), and immunostimulating (*6*) properties.

The soybean seed also contains naturally occurring bioactive peptides and proteins such as Bowman Birk inhibitor (*7*), Kunitz trypsin inhibitor (*8*), lectin (*9*) and lunasin (*10*).

The objective of this chapter is to review the chemistry of Bowman-Birk inhibitor, Kunitz trypsin inhibitor, lunasin and β-conglycinin in soybean as they present promising impact on health. Techniques on their isolation, purification and characterization are also presented.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Bowman-Birk Inhibitor

Soybean Bowman- Birk inhibitor (BBI) is composed of 71 amino acid residues with a theoretical molecular mass of 7975 Da (*11*). The structure and 3D representation of BBI is presented in Figure 1. Losso et al. (*12*) isolated BBI and found a molecular mass of 9857 Da when soybean was analyzed using matrix assisted light desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS). The isoelectric point of BBI ranges from pH 4.0 to 4.2 (*13*) with an extinction coefficient of 4.3 at 280 nm (*14*). It is also rich in cysteine residues which accounts for as much as 20% of its total amino acids. The presence of large amounts of cysteine in the BBI structure makes it a very resistant peptide towards denaturation. This is attributed to its seven disulfide bonds between residues 9 and 24; 14 and 22; 8 and 62; 12 and 58; 36 and 51; 32 and 39; and 41 and 49. The native structure of BBI is composed of 61% β-sheet, 38% unordered structure, 1% β-turn and no helices while its reduced form is 53% β-sheet, 5% β-turn and 42% unordered (*15*). Fratali and Steiner (*16*) reported that BBI does not contain helical structure or tryptophan amino acid. X-ray crystallography and nuclear magnetic resonance of BBI revealed that its tertiary structure is composed of two domains, the antitryptic and antichymotryptic domains (*17*). The first domain has a preference towards positively charged amino acids while the second domain prefers large nonpolar side chains (*18*). BBI forms a 1:1 complex with trypsin and chymotrypsin involving the Lys_{16} and Ser_{17} of the amino terminal and Leu_{43} and Ser_{44} of the carboxyl end, respectively. It is an inhibitor of elastase, cathepsin G, mast cell chymase and trypsin and a reversible inhibitor of serine proteases with chymotrypsin-like specificity (*18*).

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

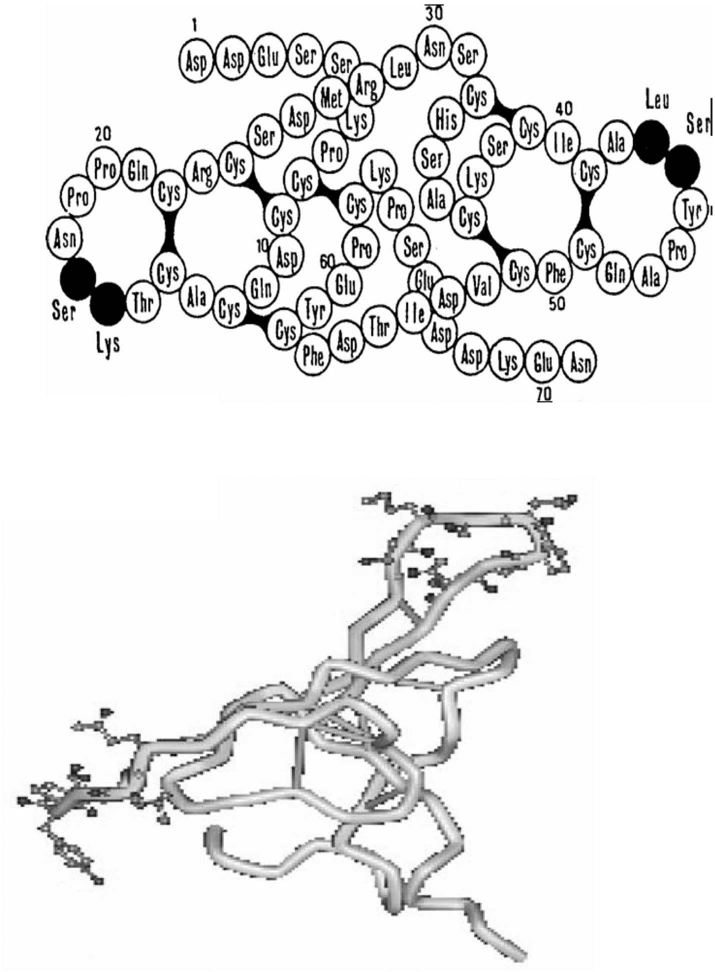

1
Asp
Asp
Glu
Ser
Ser
Met
Arg
Leu
30
Asn
Ser
Cys
His
Cys
40
Ile
Ala
Leu
Ser
20
Pro
Gln
Pro
Asn
Ser
Lys
Thr
Cys
Cys
Arg
Cys
Ser
Asp
Lys
Pro
Cys
Cys
Lys
Pro
Ser
Ala
Ser
Lys
Cys
Cys
Tyr
Pro
Ala
Gln
Cys
Cys
10
Asp
60
Pro
Glu
Ser
Gln
Ala
Cys
Tyr
Cys
Phe
Asp
Thr
Ile
Glu
Asp
Asp
Val
Cys
Phe
50
Asp
Lys
Glu
Asn
70

Figure 1. Top: Primary structure of soybean BBI. The active residues are shown in black and the disulfide bridges are also indicated in black (13). Bottom: Three-dimensional structure of soybean BBI (95).

BBI was first isolated from soybean seeds by Bowman in 1946 and further characterized by Birk in 1947, hence the name Bowman-Birk inhibitor. Hammond et al. (*19*) showed that the mRNA for BBI accumulates during early stages of embryogeny and it is composed of about 450 nucleotides. Bowman pioneered the isolation of BBI from soybeans where he used a precipitation method involving an acetone-insoluble powder. Today, the most commonly used technique of isolating BBI from soybean is the one proposed by Odani and Ikenaka (*20*). In this method, 60% ethanol is used to extract BBI from soybean at room temperature. This step is then followed by the addition of cold acetone, filtration and adjustment to pH 5.3 and subsequent acetone precipitation. The precipitate is then dissolved in water and dialyzed against distilled water. The dialyzed solution of BBI is

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

further purified using ion-exchange chromatography at pH 4.0 using a column packed with carboxymethylcellulose. Bound BBI is then eluted with a salt gradient ranging from 0 to 0.5 M NaCl. To produce purified BBI, an additional ion exchange chromatography, using diethylaminoethyl (DEAE) column at pH 6.5, is used. Since the method used by Odani and Ikenaka (*20*) has a downside of very low yield, other scientists have used different methods to purify BBI from soybean seeds. Yeboah et al. (*21*) were able to obtain 37 mg BBI from 200 g starting material with at least 95% purity. The method of isolation involved the combination of ammonium sulfate precipitation, hydrophobic interaction chromatography and gel filtration chromatography. Gladysheva et al. (*22*) were able to quantify BBI at a level of 2.27 mg per gram of soybean flour and 0.23 mg BBI in heated soybean flour using titration with α-chymotrypsin. In this study, they showed that freshly milled soybean flour is the best source of BBI.

BBI is classically identified using gel electrophoresis by virtue of its molecular mass which is approximately 8000 Da. Measurement of trypsin and chymotrypsin inhibition has also been a classical method of identifying the presence of BBI in soybean and soybean products. In food and human body fluids the most commonly used method to quantify BBI, is an immunoassay method based on monoclonal and polyclonal antibodies. Wan et al. (*23*) developed a method of determining BBI in human urine samples using 4 kinds of monoclonal antibodies (3B6, 3E3, 4H8 and 5G2) produced from a mouse immunized with reductively modified BBI. Their results suggested that three of the antibodies were able to react with BBI metabolites in urine samples. Brandon and Friedman (*24*) developed an immunoassay for the determination of BBI and the results of their experiments showed that BBI contained two epitopes. They showed excellent agreement among ELISA results; antibody 238 recognized the native structure of BBI and did not demonstrate binding towards inhibitors from lima beans and chickpeas. Furthermore, the antibody developed showed usefulness in immunoaffinity chromatography and blotting methods. Gladysheva et al. (*22*) used ELISA and immunoaffinity chromatography to quantify BBI from different cultivars. Brandon et al. (*25*) used a monoclonal antibody-based sandwich ELISA to detect levels of BBI in infant formulas. They showed that the method has a limit of quantification of 1 ng/mL and that it was also capable of detecting BBI in soybean germplasm with atypically low BBI content.

BBI is being recognized as a potential chemopreventive agent, particularly in the treatment and prevention of certain type of chronic diseases. BBI has been reported to be capable of inhibiting proteases released from inflammation mediating cells and suppressed superoxide anion radical secretion from immunocytes. A study showed that BBI concentrate can have a beneficial effect on dextran sulfate sodium-treated mice and may be useful in the treatment of human inflammatory bowel diseases such as ulcerative colitis (*26*). In a recent study involving patients with active ulcerative colitis, BBI concentrate with 800 chymotrypsin inhibitory units showed a potential benefit over placebo for achieving both clinical response and induction of remission with no apparent toxicity (*27*). Mice treated with spermine-BBI lowered their esterified cholesterol in the aortas in 9% in comparison to controls. Purified BBI and spermine-BBI reduced total cholesterol levels in the blood by 15.5% and 33.3%, respectively.

It was also found that spermine-BBI can prevent lung carcinogenesis without detectable toxic effects (*28*). Arbogast et al. (*29*) found that adult mice dietary supplementation with BBIC (1% during hind limb unloading for up to 12 days) protects skeletal muscle during prolonged unloading, promoting redox homeostasis in muscle fibers and blunting atrophy-induced weakness. It was also found that BBIC directly interacts with reactive oxygen species and inhibits serine protease activity in vitro.

Other reported properties of BBI are its capability to inhibit matrix metalloproteinase activity (*12*), in vitro inhibition of the activation of pro-matrix metalloproteinases 1 and 9 (*30*) inhibition of proliferation of breast cancer cells (MCF-7) and hepatoma cells (Hep G2) (*7*) and inhibition of HIV-1 reverse transcriptase (*7*). In a mouse xenograft model, BBI treatment reduced the relative tumor weight and restored the level of connexin 43 mRNA in a dose-dependent manner, an important factor for cancer prevention (*31*).

Currently, BBI concentrate (BBIC) is in Phase II human clinical trial as a potential chemopreventive agent against head and neck cancer (precancerous/nonmalignant conditions) and in Phase I human clinical trial for unspecified adult solid tumor (www.clinicaltrials.gov). As a human drug, a pilot study consisting of a Phase I and Phase IIa trials have been completed for patients with oral leukoplakia. Results from these studies showed that BBIC is not toxic at 200 to 1066 chymotrypsin inhibitor units per day causing a statistical significant reduction of total leukoplakia lesion size that was linearly correlated with dose (*32*). Based on this, a Phase IIb trial is ongoing which is thought to be a Phase III trial for the treatment of oral leukoplakia and a Phase II trial for oral cancer prevention (*32*). Phase I and II trials were completed for possible treatment of benign prostatic hyperplasia. In these latter human trials, no toxicity was observed at dosage levels of 100 to 800 chymotrypsin inhibitor units per day with a statistically significant decrease in prostate serum antigen levels (*32*). In addition, BBIC human trial for treatment of gingivitis has been completed and showed that BBIC treatment results in significant decrease in lactase-like activity in gingivical crevicular fluid which is attributed to causation of periodontal disease (*33*). It has also been shown that BBI and BBIC are not genotoxic, teratogenic or oncogenic and in addition drug induced toxicity has not been observed in animals for both forms of BBI (*32*). In summary, the structure and chemistry of BBI from soybean are known; however, there is still a need to further study its biological properties for the public to fully take advantage of its potential as a chemopreventive agent.

Kunitz Trypsin Inhibitor

Figure 2A presents the ribbon diagram and 3D structure of Kunitz trypsin inhibitor (KTI). The molecular mass of KTI is 21.5 kDa and it is composed of 181 amino acid residues. It was first isolated in soybean seeds by Kunitz (*34*). The partial structure in complex with porcine pancreatic trypsin was reported first in 1974 (*35*, *36*). Later, Song and Suh (*37*) reported a refined structure of KTI complexed with porcine trypsin as shown in Figure 2B. The overall structure of KTI is that of a sphere of about 3-5 nm in diameter, consisting of 12 criss-crossing

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

β-sheets oriented in antiparallel fashion stabilized by hydrophobic side chains (*38*). It has two disulfide bridges involving Cys39-Cys86 and Cys136-Cys145 (*39*) which might explain its unusual stability towards chemical and thermal denaturation. Although disulfide bonds are not located near the active site of KTI, they play a crucial role in stabilizing the protein structure, necessary to sustain enzymatic activity. KTI has been found to have eight distinguishable isoforms, namely T*ia* and T*ib* (*40*), T*ic* (*41*), *ti*-null type (*42*), T*id* (*43*), T*ie* (*44*, *45*), T*if* (*46*) and T*ib*^{i5} (*47*).

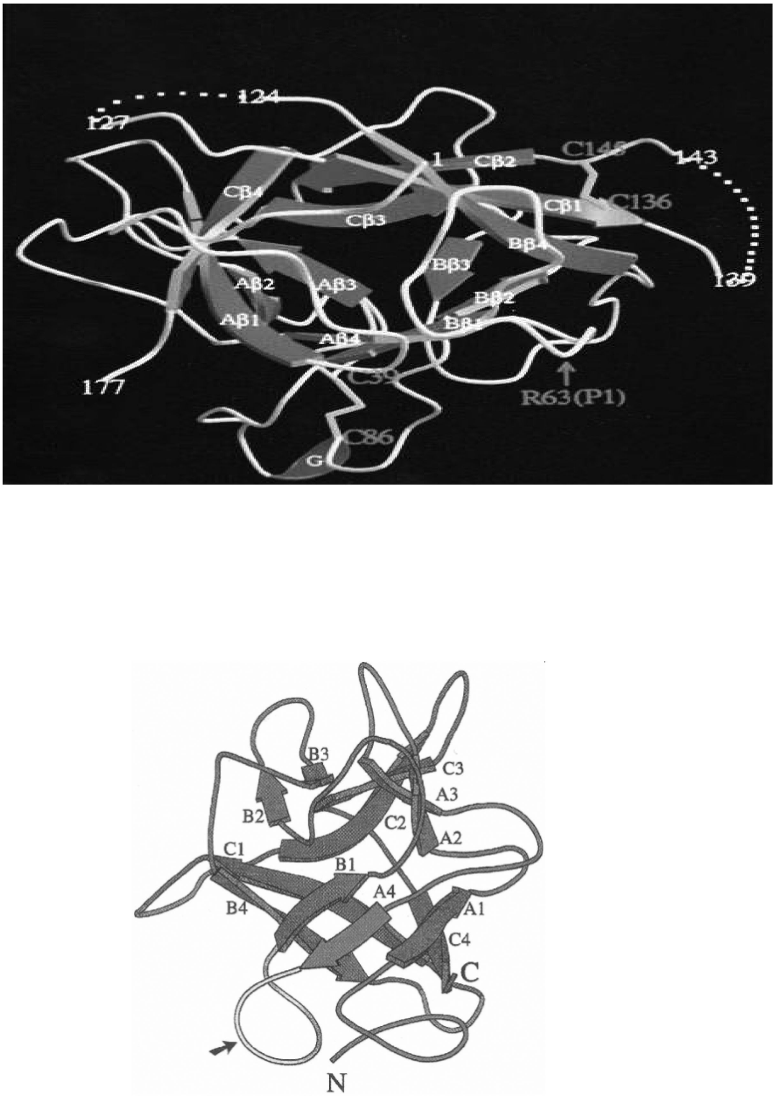

124
127
1
Cβ2
C145
143
Cβ4
Cβ3
Cβ1
C136
Bβ4
Bβ3
Aβ2
Aβ3
Bβ2
139
Aβ1
Bβ1
Aβ4
177
C39
R63(P1)
C86
G
B3
C3
A3
B2
C2
A2
C1
B1
A4
A1
B4
C4
C
N

Figure 2. Top: Ribbon diagram of soybean Kunitz trypsin inhibitor (37). Bottom: A ribbon representation of the overall fold of soybean Kunitz trypin inhibitor. The pseudo threefold axis corresponds to the axis of the barrel (91).

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Kunitz reported the isolation of KTI from cold-processed defatted soybean meal (*34*). He found out that the crystalline protein precipitates when heated in 2.5% trichloroacetic acid, and have a minimum solubility in water at pH range of 4.2 to 4.8. He also showed that it lacks carbohydrate residues and it is a globulin type protein soluble in dilute acid, alkali or salt solutions. Duranti et al. (*48*) reported a fast and simple method for the extraction and purification of KTI from soybean seeds. They heated soybean seeds at 60 °C for 90 min and the aqueous medium was loaded into an affinity chromatography column with immobilized trypsin. Retained KTI was then eluted with 0.01N HCl and freeze dried. They were able to obtain 0.7 mg of the inhibitor with specific trypsin inhibitory units of 11,430 TIU/mg protein. Sugarawa et al. (*49*) used a mixture of Tris-HCl buffer containing anti-protease enzyme in 0.2 M NaCl and 20% glycerol to extract KTI from frozen cotyledons. Then, the extract was subjected to centrifugation and ammonium sulfate fractionation. In order to purify KTI, they used Sephadex G-75 gel filtration column followed by Poros HQ/M anion exchange chromatography. Trypsin inhibitory activity was used to screen fractions containing KTI. In addition, they determined the activity for KTI using native-PAGE gels stained with tetrazoitized *o*-dianisidine. Shakiba et al. (*50*) used affinity column with immobilized trypsin to isolate KTI from aqueous extract of defatted soybean meal. The purification was further achieved by subsequent anion exchange chromatography on a DEAE Sepharose Fast Flow Column. The concentration of the purified KTI was measured at 280 nm and reducing SDS-PAGE was used to estimate molecular mass and purity of isolated KTI. The use of affinity chromatography with trypsin bound to matrix presents a simple and inexpensive method of purifying KTI from soybean seeds while the methods used by Sugarawa et al. (*49*) used a repeated excision of KTI from native-PAGE and SDS-PAGE gels which is definitely time consuming and needs special skills to carry out .

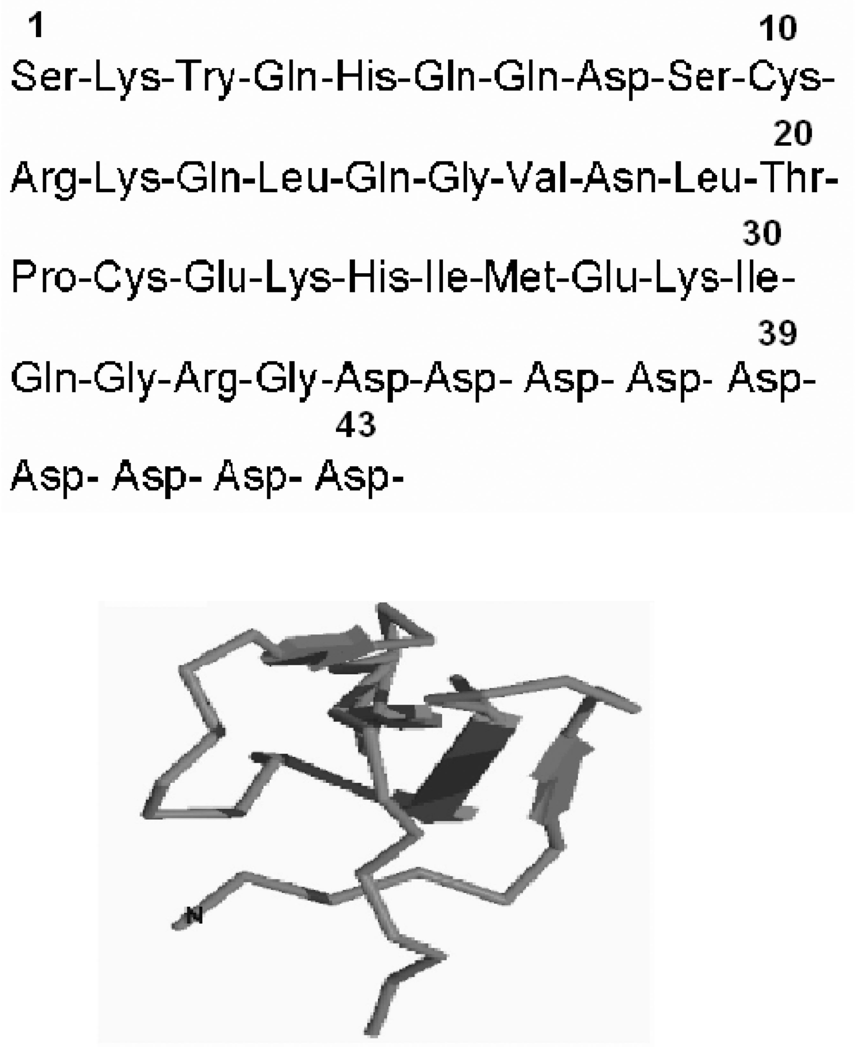
Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Wang et al. (*47*, *51*) performed Western blot analysis using anti-trypsin soybean inhibitor rabbit polyclonal antiserum to identify KTI in different soybean lines. Immunoassay is the most commonly used technique in determining the amount of KTI present in soy and soy formulations. Brandon and Bates (*52*) demonstrated the interaction of monoclonal antibodies with the three Kunitz trypsin inhibitor isoforms designated as Ti^a, Ti^b and Ti^c. They used an antibody that was derived from mice previously inoculated with KTI isoform and then purified by ammonium sulfate fractionation and DEAE cellulose chromatography. Brandon et al. (*25*) presented a monoclonal antibody-based sandwich enzyme-linked immunosorbent assays for the quantification of KTI and BBI in infant formulas. The method had a detection limit of 3 ng/mL for KTI.

Studies on the possible role of KTI in disease prevention have shown that KTI possess certain biological activities. Kobayashi et al. (*53*) showed that KTI abrogates LPS-induced up-regulation of TNF-α mRNA and protein expression in gingival fibroblasts. Also, they showed that KTI is capable of inhibiting production of interleukin-1β and interleukin-6 cytokines in LPS-induced gingival fibroblasts. Another study by Kobayashi et al. (*54*) showed that KTI has the same effect on TNF-α mRNA expression and production of interleukins-1β and 6 cytokines in UV-exposed primary human keratinocytes.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Furthermore, they showed that KTI specifically inhibited UV-induced activation of extracellular-signal regulated kinase, c-Jun N-terminal kinase and p38 but not Akt. Kobayashi et al. (*55*) showed that KTI was able to suppress ovarian cancer cell invasion by blocking urokinase up-regulation. A recent study by Shakiba et al. (*50*) showed that KTI has an anti-angiogenic effect on human umbilical vein endothelial cells without any toxic effects up to a concentration of 600 μg/mL. A recent patent describes the composition of skin care products containing KTI that can be applied topically and reduce the risk of UV-induced cutaneous tumors (*56*).



1 10
Ser-Lys-Try-Gln-His-Gln-Gln-Asp-Ser-Cys-
20
Arg-Lys-Gln-Leu-Gln-Gly-Val-Asn-Leu-Thr-
30
Pro-Cys-Glu-Lys-His-Ile-Met-Glu-Lys-Ile-
39
Gln-Gly-Arg-Gly-Asp-Asp- Asp- Asp- Asp-
43
Asp- Asp- Asp- Asp-

Figure 3. Top: The 43-amino acid sequence of soybean lunasin. Bottom: Predicted secondary structure of lunasin with helix and structural homology to conserved region of chromatin-binding proteins.

In summary, the structure of KTI and methods by which it can be characterized in soybean are known and its reported biological properties create a niche as another chemopreventive agent from soybean. Regarding safety, two studies using neonatal pigs as a model, chosen for its similarities to human digestive physiology and anatomy, have demonstrated no toxicity with approximately 500 mg KTI/100 g dry matter incorporated into the diet (*57*, *58*).

Lunasin

Lunasin is a chemopreventive peptide originally isolated from soybean. It is composed of 43 amino acid residues and contains nine aspartic acid residues on its carboxyl end, a cell adhesion motif composed of arginine-glycine-aspartic acid residues and a predicted helix with structural homology to a conserved region of chromatin binding proteins (*59*, *60*). It has an approximate molecular mass of 5500 Da and a predicted helix with structural homology to a conserved region of chromatin-binding proteins (*2*, *59*). The sequence of amino acid and the predicted helix structure of lunasin are shown in Figure 3. In order to fully understand the biological properties of lunasin, there is a need to study the different levels of its chemical structure.

Lunasin has been isolated from soybean, barley, wheat and other plant sources (*59–63*). Jeong et al. (*64*) extracted lunasin from soybean seed using phosphate buffered saline at pH 7.4 for 48 h at 4°C. The extract was then dialyzed for 24 h at 4 °C in distilled water, after which the dialyzed extract was centrifuged at 12000g for 30 min and the precipitate re-extracted using 10 mL of the extracting buffer. The extract was further purified using ion exchange chromatography on Biogel resin AG and the bound lunasin was eluted using 0.7 M NaCl. The concentrated sample from ion exchange was further purified using immunoaffinity column chromatography with rabbit polyclonal antibody against the carboxyl end of lunasin and the bound lunasin was eluted with 20 mL of 0.2 M glycine-HCl. In our laboratory, we were able to purify lunasin from defatted soybean flour using a combination of ion-exchange chromatography, ultrafiltration and gel filtration chromatography (*10*). The ratio of extracted lunasin with distilled-deionized water to defatted soybean flour was 5:1. The mixture was then stirred at room temperature for 90 min and centrifuged for 10 min at 12000g. The supernatant was pooled and filtered using 0.22 µm filter. The filtered supernatant was loaded into DEAE ion exchange chromatography and bound lunasin was eluted using 0.15 M NaCl. Fractions from DEAE ion exchange chromatography containing high concentrations of lunasin were pooled and concentrated using ultraflitration membrane with a molecular weight cut-off of 3 kDa. The retentate was applied in size exclusion chromatography with 25 kDa size exclusion limit using 20 mM Tris HCl buffer, pH 7.5 with 0.15 M NaCl as eluting buffer. Our results showed that most of the lunasin eluted from the size exclusion column after 1.5 void volumes. Figure 4 summarizes the previously described isolation and purification procedure for lunasin from soybean. Modification of this procedure using a combination of ultrafiltration and microcentrifugation resulted in ~90% lunasin purity.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

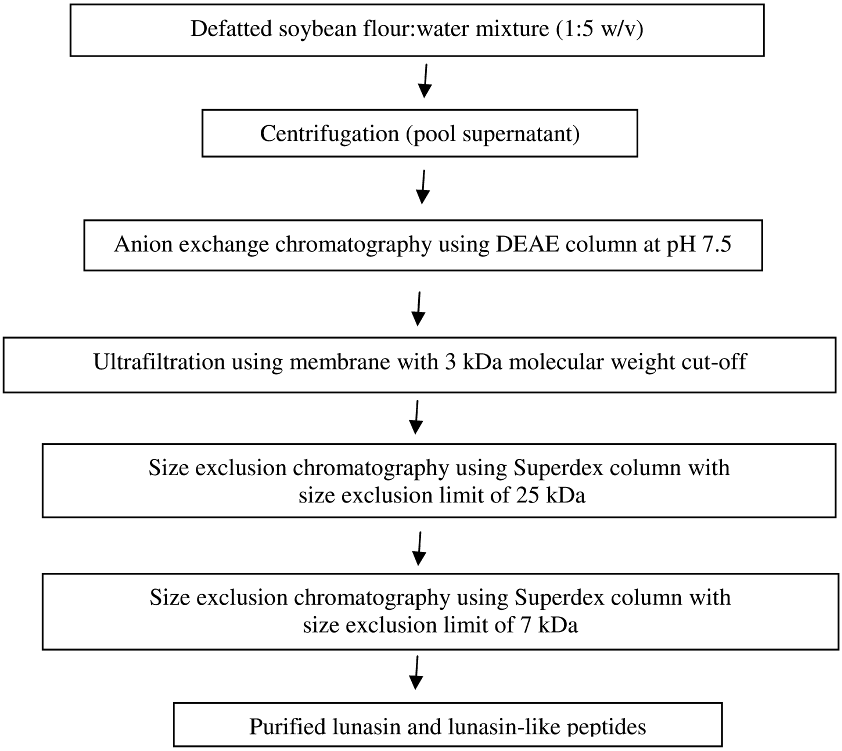

Defatted soybean flour:water mixture (1:5 w/v)
Centrifugation (pool supernatant)
Anion exchange chromatography using DEAE column at pH 7.5
Ultrafiltration using membrane with 3 kDa molecular weight cut-off
Size exclusion chromatography using Superdex column with size exclusion limit of 25 kDa
Size exclusion chromatography using Superdex column with size exclusion limit of 7 kDa
Purified lunasin and lunasin-like peptides

Figure 4. Summary of isolation and purification methods for soybean lunasin.

The identity and quantity of lunasin was established using several methods. Jeong et al. (*64*) established the identity of lunasin using SDS-PAGE, Western blot and MALDI peptide mass mapping. Dia et al. (*10*) confirmed the presence of lunasin like peptides from defatted soybean flour using SDS-PAGE, Western blot, MALDI, and LC-MS/MS. They showed that there were three peptides that have positive immunoreactivity towards lunasin monoclonal antibody corresponding to 5, 8 and 14 kDa. The concentrations of lunasin in soybean and soybean products were quantified using enzyme linked immunosorbent assay and Western blot. Jeong et al. (*60*) quantified lunasin using a standard curve derived from the intensities of different concentrations of synthetic lunasin. They showed that the method has a detection limit of 15 ng lunasin and the curve of signal intensity versus the amount of lunasin has a high correlation coefficient of 0.95. De Mejia et al. (*65*) used ELISA and Western blot to quantify lunasin in different soybean genotypes, commercial soy protein and isoflavone products. They showed very high correlation between synthetic lunasin concentration and absorbance at 405 nm for ELISA and immunoblot band intensity with correlation coefficients of 0.963 and 0.9625, respectively. They were also able to show that ELISA has better sensitivity with a detection limit of 8 ng/mL than Western blot with a detection limit of 8.5 µg/mL. Hernandez-Ledesma et al. (*66*) quantified the amount of lunasin and Bowman-Birk inhibitor in U.S. commercial soy foods including soy milk, soy-based infant formula, tofu, bean curd, soybean cake, tempeh, natto, miso and su-jae. Their results showed that the two peptides are present in most of the samples analyzed in varying concentrations depending on the soybean variety and manufacturing process. They also found out that the two peptides are absent in fermented foods natto and tempeh suggesting that fermentation process destroy both peptides. Our laboratory quantified the amount of lunasin in soybean seeds believed to containing no BBI. The concentration of lunasin in BBI-null soybean seeds ranged from 3.34 ± 0.32 to 16.74 ± 0.95 ng lunasin per mg seed (Table I). These data show that lunasin is still produced in soybean seed even in the absence of BBI, however, these concentrations are extremely low when compared to conventional soybean seeds expressing BBI.

Galvez et al. (*59*) showed that synthetic lunasin was capable of inducing apoptosis, mostly on cells undergoing transformation, by preventing histone acetylation. Lam et al. (*67*) showed that lunasin was able to suppress transformation of mammalian cells induced by an oncogene (E1A). They also showed that lunasin has no effect on growth of immortalized (nontumorigenic) and established cancer cells. Jeong et al. (*64*) first showed that lunasin from soybean possess biological activity affecting the process of histone acetylation-deacetylation. They showed that lunasin purified from soybean suppressed colony formation induced by the *ras*-oncogene and inhibit core H3-histone acetylation. Jeong (*61*) showed that lunasin from different Korean soybean varieties is capable of inhibiting core histone H3- and H4-acetylation. More recently, Dia et al. (*10*) showed for the first time the anti-inflammatory properties of lunasin purified from defatted soybean flour through inhibition of prostaglandin E_2/cyclooxygenase-2 and nitric oxide/inducible nitric oxide synthase pathways. Recent studies have shown that lunasin inhibit inflammation in lipopolysaccharide-induced RAW 264.7 through inhibition of NF-κB pathway and release of pro-inflammatory cytokines interleukin-6, interleukin-1β and tumor necrosis factor-α (*68*, *69*). Moreover, Hernandez-Ledesma have shown that lunasin inhibits linoleic acid oxidation and acts as free radical scavenger (*69*).An important aspect just recently described is the potential biological implications related to the arginine-glycine-aspartate (RGD) motif in lunasin in promoting apoptosis in cancer cells (*70*). In addition, in our laboratory we have demonstrated, for the first time, the presence of lunasin in blood of men extracted 30 min and 1 h after 5 days of consumption of soybean products (*71*). This means that lunasin is bioavailable and can be absorbed by the gastrointestinal system to exert its biological activity in target tissues. In summary, significant advances have been made on the structure, chemistry and biological function of lunasin from soybean. This represents an important opportunity to study further the potential of lunasin as a bioactive compound against various disease conditions such as inflammation, cardiovascular risk and cancer.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Table I. Lunasin concentration of soybean genotypes void of Bowman-Birk inhibitor

PI number	*Cultivar name*	*Scientific Name*	*Lunasin, ng/mg seed**
573055	G2119	*Glycine latifolia*	3.32 ± 0.34
591602	G2740	*Glycine tabacina*	3.55 ± 0.13
591597	G2118	*Glycine latifolia*	3.72 ± 0.60
595821	G1257	*Glycine tabacina*	3.77 ± 0.22
509486	G1555	*Glycine microphylla*	3.88 ± 0.17
505166	G1849	*Glycine curvata*	4.07 ± 0.20
599406	G1553	*Glycine cyrtoloba*	4.14 ± 0.21
599417	G2490	*Glycine cyrtoloba*	4.25 ± 0.08
591610	G2954	*Glycine tomentella*	4.34 ± 0.49
563889	G2263	*Glycine tabacina*	4.35 ± 0.18
505196	G1830	*Glycine microphylla*	4.40 ± 0.31
505234	G1820	*Glycine tomentella*	4.43 ± 0.34
509496	G1262	*Glycine tabacina*	4.51 ± 0.63
483223	G2406	*Glycine tomentella*	4.57 ± 0.18
573078	G1691	*Glycine tomentella*	4.58 ± 0.95
559310	G2340	*Glycine microphylla*	4.61 ± 0.22
604471	G2104	*Glycine cyrtoloba*	4.78 ± 0.39
559297	G2121	*Glycine latifolia*	4.94 ± 0.22
446994	G1365	*Glycine tomentella*	5.06 ± 0.40
559346	G2285	*Glycine tabacina*	5.10 ± 0.20
440964	G1186	*Glycine cyrtoloba*	5.49 ± 0.45
563891	G2287	*Glycine tabacina*	5.75 ± 0.69
446993	G1364	*Glycine tomentella*	6.23 ± 0.57
440962	G1184	*Glycine cyrtoloba*	6.32 ± 0.30
339661	G1138	*Glycine tabacina*	9.43 ± 0.38
272099	G1860	*Glycine tabacina*	10.65 ± 0.70
253238	G1343	*Glycine latifolia*	10.90 ± 0.51
339664	G1143	*Glycine microphylla*	11.93 ± 0.74

Continued on next page.

Table I. (Continued). Lunasin concentration of soybean genotypes void of Bowman-Birk inhibitor

PI number	*Cultivar name*	*Scientific Name*	*Lunasin, ng/mg seed*[*]
319696	G1694	*Glycine latifolia*	12.63 ± 0.96
373983	G1424	*Glycine tabacina*	13.35 ± 1.07
321393	G2545	*Glycine latifolia*	13.48 ± 1.34
248253	G2762	*Glycine tabacina*	14.84 ± 2.78
339665	G1311	*Glycine microphylla*	15.92 ± 0.51
373993	G1236	*Glycine cyrtoloba*	16.55 ± 0.83
193232	G2763	*Glycine tabacina*	16.74 ± 0.95

[*] Mean of three trials ± standard deviation.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

β-Conglycinin

One of the storage proteins present in soybean seeds is β-conglycinin (BC). It is a vicilin storage protein with a molecular mass of 150-200 kDa (*70*). BC is composed of three subunits called α, α′ and β (*73*). Alpha and α′ subunits are composed of core regions with a high degree of homology and extension regions exhibiting lower homologies. On the other hand, the β subunit is composed only of a core region with 75.5 and 71.4% homology with α and α′ core regions, respectively. Utsumi et al. (*74*) reported that the three subunits are associated through strong hydrophobic and hydrogen bonding. The crystallized structure of α and α′ subunits of BC are presented in Figure 5. BC is a glycoprotein and undergoes association-dissociation phenomenon in response to changes in ionic strength and pH. At neutral pH and ionic strength of > 0.5, it is a 7S-form globulin, but at ionic strength of < 0.2, it forms an aggregate to become a 9S (*75*). Maruyama et al. (*76*) demonstrated the crystal structure of native and recombinant β-subunit homotrimer and found that the quaternary structure of the β-subunit is very similar to 7S globulins of jack bean and kidney bean. In 2004, the crystal structure of the core region of α′ subunit of BC with construction of deletion mutants was shown by Maruyama et al. (*77*). Maruyama et al. (*78*) showed that the core regions of α, α′ and β subunits are homologous with each other with α and α′ 90%, α and β 76% and α′ and β 76%.

In our laboratory (*72*), isolation of BC was achieved by modifying the method reported by Nagano et al. (*79*). Briefly, 100 g of defatted soybean flour were suspended in 1.5 L of distilled water, followed by pH adjustment to 7.5 with 2 N NaOH and centrifugation of the solution for 30 min at 9000g. Sodium bisulfite was then added and the pH was adjusted again to 5.9 with 2 N HCl and the mixture stored overnight at 4 °C. After storage, the mixture was centrifuged at 6500g for 20 min at 4 °C and salt was added to the supernatant to adjust the salt concentration to 0.25 M. The pH was adjusted to 5.0 by addition of 2N HCl and

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

stored at 4 °C for 1 h. The mixture was then centrifuged at 9000g for 30 min at 4 °C, and two volumes of ice cold distilled water were added to the supernatant. After pH adjustment to 4.8 with 2 N HCl, the mixture was centrifuged at 6500g for 20 min at 4 °C and the precipitate collected. The precipitate was then washed twice with cold distilled water and resuspended in 25 mL water and designated as purified BC with > 80% purity. Hou and Chang (*80*) further purified a crude BC extract from defatted soybean flour by chromatography. They used an affinity column with concanavalin A ligand and the bound BC was eluted with Tris-HCl buffer containing 0.1 M α-methyl-D-mannopyranoside. Fractions with high concentration of BC, as shown in SDS-PAGE, were pooled and concentrated via ultrafiltration using a 10 kDa cut off membrane. The retentate was introduced through a Sephacryl S-300 superfine gel filtration column and the elution of BC was monitored at 280 nm and further confirmed by SDS-PAGE. BC is considered a good source of bioactive peptides. The bioactive peptides are derived either by enzyme digestion or through fermentation. Table II summarizes the recently derived peptides from BC and their corresponding biological activities. Wang et al. (*72*) demonstrated that BCs are among the major protein components that inhibit leukemia cell growth in vitro. They showed that soybean genotypes with higher concentration of BC have lower IC_{50} values than soybean genotypes with lower BC content towards inhibition of L1210 leukemia cell growth. They also reported an IC_{50} of 4.2 mg/mL for BC -enriched soybean genotype hydrolysates and an IC_{50} of 5.6 mg/mL for hydrolysates from soybean genotype with lower BC content. There was less cytotoxicity towards leukemia cells when they used purified BC hydrolysates as compared to hydrolyzed soybean flour with higher BC content, suggesting the additional effect of other bioactive components in soybean flour such as isoflavones. Zuo et al. (*81*) showed that pepsin digestion of soybean BC with molecular mass ranging from 693.32 to 1829.55 Da resulted in the stimulation of growth of Bifidobacteria. Furthermore, they showed that mice fed with the most active fraction isolated from pepsin-treated conglycinin resulted in the significant decrease in cecal pH and enterococci count concomitant with increase level of IgA and activity of β-galactosidase. Other studies showed anti-microbial properties of hydrolysates derived from BC. Shen et al. (*82*) reported that pepsin-hydrolysate conglycinin inhibited the growth of *Escherichia coli* O_{138} keeping the mice healthy following oral administration of *Escherichia coli* O_{138} infection and maintaining the balance of active microbial community in mice gastrointestinal tract. Yang et al. (*83*) demonstrated the capability of alcalase hydrolysates of BC to inhibit translocation of *Salmonella thyphimurium* into intestinal epithelial cells. They also concluded that peptides derived from alcalase hydrolysates of BC with molecular mass of 10-20 kDa were more effective than lower molecular mass peptides. Another study showed that soymorphins derived from BC with amino acid sequence of YPFVV and YPFV possessed anxiolytic activity (*84*). Martinez-Villaluenga et al. (*85*) reported that alcalase hydrolysates of soybean from genotypes containing higher concentration of BC have higher inhibition rate of lipid accumulation in 3T3-L1 adipocytes than alcalase hydrolysates with lower BC content. The suggested mechanism of action was through the inhibition of fatty acid synthase (*86*). Peptides from highly purified and isoflavone-free BC decreased triglyceride synthesis and

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

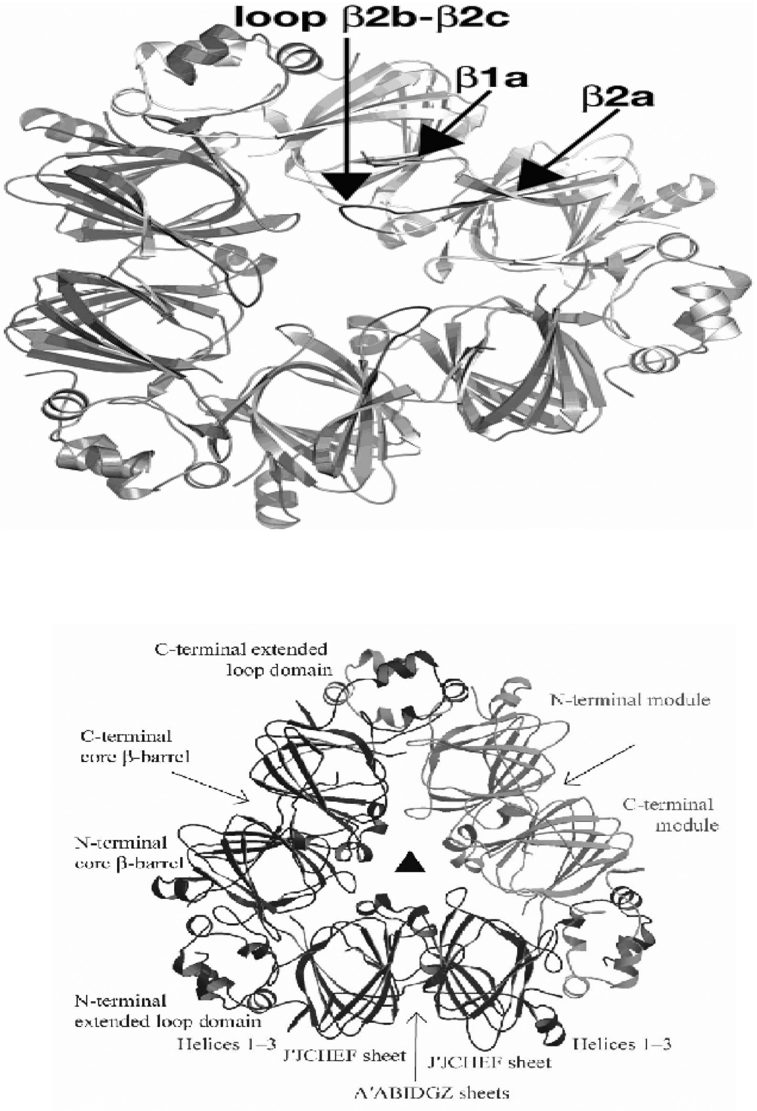

loop β2b-β2c
β1a
β2a
C-terminal extended loop domain
N-terminal module
C-terminal core β-barrel
C-terminal module
N-terminal core β-barrel
N-terminal extended loop domain
Helices 1–3
J'JCHEF sheet
J'JCHEF sheet
Helices 1–3
A'ABIDGZ sheets

Figure 5. Top: Ribbon diagram shows the overall secondary structure of the soybean β-conglycinin α-subunit homotrimer. β-strands are shown as barrels and α-helices as coils (93). Bottom: Ribbon diagram of α_c' subunits of soybean β-conglycinin as seen along a molecular threefold axis (black triangle) (77).

secretion of lipoprotein particles containing ApoB-100 from HepG2 cells with changes in the gene expression profile that are consistent with the reduction of hypertriglyceridemia (*87*). A process for the extraction, purification and enzymatic modification of BC α′-subunit was successfully developed for use as a hypocholesterolemizing agent (*88*).

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Table II. Recently derived bioactive peptides/hydrolysates from β-conglycinin

Hydrolysates/Peptide	*Activity*	*Analysis/Techniques Employed*	*Reference(s)*
Soymorphin-5 (YPFVV) Soymorphin-6 (YPFVVN) Soymorphin-7 (YPFVVNA)	Opiod	Guinea pig ileum and mouse vas deferens assay; Fmoc strategy	(*84*)
Pepsin treated conglycinin (693.32-1829.55 Da)	Stimulated growth of Bifidobacteria in vitro; increased sIgA level and β-galactosidase activity; inhibited the growth of *E. coli* O138	Pepsin hydrolysis; size exclusion chromatography (SEC); dialysis; MALDI-TOF	(*81*, *83*)
Alcalase hydrolysates (10-20 kDa)	Resisted *Salmonella thypimurium* Penetration into intestinal epithelial cells	Alcalase hydrolysis; SEC-HPLC; ultrafiltration	(*83*)
Soymetide-4 (MITL)	Immunostimulating; anti-alopecia	fMOC; RP-HPLC	(*94*)
Soymetide-13 (MIT-LAIPVNKPGR)	Stimulated phagocytosis in human neutrophils	Trypsin digestion; DEAE-cellulose column chromatography; RP-HPLC	(*6*)
FEITPEKNPQ IETWNPNNKP VFDGEL	Inhibition of human topoisomerase II	Co-immunoprecipitation; Cap-LC Micromass Q-tof	(*92*)

In summary, the chemistry of β-conglycinin is well understood and its hydrolysis, using enzymes or via fermentation, results in the production of peptides with important biological properties. Figure 6 presents a general illustration of the isolation, purification and characterization of biologically active peptides from soybean. Figure 7 presents the amino acid sequence of the three peptides, BBI, lunasin and Kunitz. They show no clear homologies in their composition.

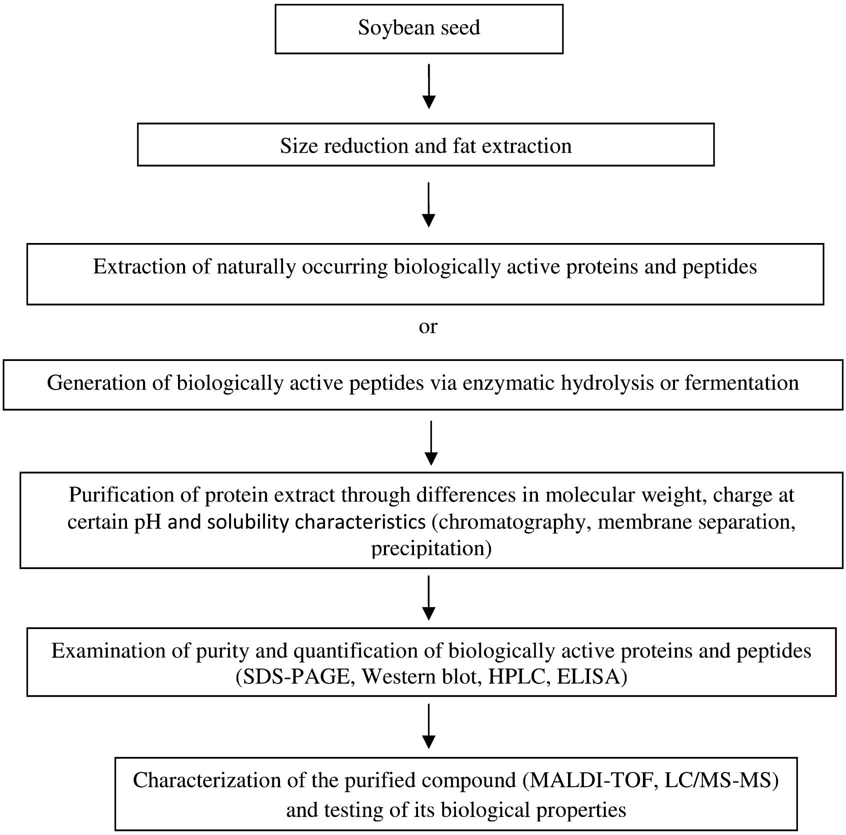

Soybean seed
Size reduction and fat extraction
Extraction of naturally occurring biologically active proteins and peptides
or
Generation of biologically active peptides via enzymatic hydrolysis or fermentation
Purification of protein extract through differences in molecular weight, charge at certain pH and solubility characteristics (chromatography, membrane separation, precipitation)
Examination of purity and quantification of biologically active proteins and peptides (SDS-PAGE, Western blot, HPLC, ELISA)
Characterization of the purified compound (MALDI-TOF, LC/MS-MS) and testing of its biological properties

Figure 6. General steps for the isolation, purification and characterization of biologically active peptides from soybean.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Bowman-Birk Inhibitor

10	20	30	40	50
MVVLKVCLVL	LFLVGGTTSA	NLRLSKLGLL	MKSDHQHSND	DESSKPCCDQ
60	70	80	90	100
CACTKSNPPQ	CRCSDMRLNS	CHSACKSCIC	ALSYPAQCFC	VDITDFCYEP
110				
CKPSEDDKEN				

Kunitz Trypsin Inhibitor

10	20	30	40	50
MKSTIFFLFL	FCAFTTSYLP	SAIADFVLDN	EGNPLENGGT	YYILSDITAF
60	70	80	90	100
GGIRAAPTGN	ERCPLTVVQS	RNELDKGIGT	IISSPYRIRF	IAEGHPLSLK
110	120	130	140	150
FDSFAVIMLC	VGIPTEWSVV	EDLPEGPAVK	IGENKDAMDG	WFRLERVSDD
160	170	180	190	200
EFNNYKLVFC	PQQAEDDKCG	DIGISIDHDD	GTRRLVVSKN	KPLVVQFQKL
210				
DKESLAKKNH	GLSRSE			

Lunasin

10	20	30	40
SKWQHQQDSC	RKQLQGVNLT	PCEKHIMEKI	QGRGDDDDDD DDD

Figure 7. Amino acid sequence of the three peptides, BBI, lunasin and Kunitz.

Conclusions

The increasing interest posed by the public and scientific community in soybean and soybean products is attributed to their biologically active compounds of which major components are proteins and peptides. While much can be learned about these proteins as isolated components, interactions with other components should be considered. An example is the protection from digestion of lunasin by BBI when soy protein is consumed orally (*89*). Synergistic interactions between peptides and other components, such as isoflavones, have also been found (*90*). We have shown that biologically active peptides and proteins in soybean can be isolated and purified by various techniques. Also, the methods involved in their characterization and quantification have shown vast improvement on sensitivity and application. These techniques are inevitable for bioactive peptides and proteins to be accurately identified and quantified for the assessment of their biological properties and further evaluation as preventive agents of disease and promoters of health and wellness. Newly developed bioactive peptides can be used as functional ingredients or in dietary supplements.

References

1. Wang, W.; de Mejia, E. G. *Compr. Rev. Food Sci. Food Saf.* **2005**, *4*, 63–78.
2. Wang, W.; Dia, V. P.; Vasconez, M.; Nelson, R. L.; Gonzalez de Mejia, E. *J. AOAC Int.* **2008**, *4*, 936–946.
3. Kuba, M.; Tana, C.; Tawata, S.; Yasuda, M. *Process Biochem.* **2005**, *40*, 2191–2196.
4. Kodera, T.; Nio, N. *J Food Sci.* **2006**, *71*, C164–C173.
5. Zhong, F.; Zhang, X.; Ma, J.; Shoemaker, C. F. *Food Res. Int.* **2007**, *40*, 756–762.
6. Tsuruki, T.; Kishi, K.; Takahashi, M.; Tanaka, M.; Matsukawa, T.; Yoshikawa, M. *FEBS Lett.* **2003**, *540*, 206–210.
7. Ho, V. S. M.; Ng, T. B. *J. Pept. Sci.* **2008**, *14*, 278–282.
8. Shakiba, Y.; Mansouri, K.; Maostafaie, A. *Fitoterapia* **2007**, *78*, 587–589.
9. de Mejia, E. G.; Prisecaru, V. I. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 425–445.
10. Dia, V. P; Wang, W.; Oh, V.; de Lumen, B. O.; Gonzalez de Mejia, E. *Food Chem.* **2009**, *114*, 108–115.
11. Birk, Y. *Int. J. Pept. Protein Res.* **1985**, *25*, 113–131.
12. Losso, J. N.; Munene, C. N.; Bansode, R. R.; Bawadi, H. A. *Biotechnol. Lett.* **2004**, *26*, 901–905.
13. Losso, J. N. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 94–118.
14. Turner, R.; Liener, I. E.; Lovrien, R. E. *Biochemistry* **1975**, *14*, 275–282.
15. Wu, Y. V.; Sessa, D. J. *J Agric. Food Chem.* **1994**, *42*, 2136–2138.
16. Fratali, V.; Steiner, R. F. *Anal. Biochem.* **1969**, *27*, 285–291.
17. Werner, M. H.; Wemmer, D. E. *Biochemistry* **1991**, *30*, 3356–3364.
18. Csaky, I.; Fekete, S. *Acta Vet. Hung.* **2004**, *52*, 299–313.
19. Hammond, R. W.; Foard, D. E.; Larkins, B. A. *J. Biol. Chem.* **1984**, *259*, 9883–9890.
20. Odani, S.; Ikenaka, T. *J. Biochem.* **1977**, *82*, 1513–1522.
21. Yeboah, N. A.; Arahira, M.; Udaka, K.; Fukuzawa, C. *Protrein Expression Purif.* **1996**, *7*, 309–314.
22. Gladysheva, I. P.; Balabushevich, N. G.; Moroz, N. A.; Larionova, N. I. *Biochemistry (Moscow)* **2000**, *65*, 198–203.
23. Wan, X. S.; Koch, C. J.; Lord, E. M.; Manzone, H.; Billings, P. C.; Donahue, J. J.; Odell, C. S.; Miller, J. H.; Schmidt, N. A.; Kennedy, A. R. *J. Immunol. Methods* **1995**, *180*, 117–130.
24. Brandon, D. L.; Friedman, M. *J. Agric. Food Chem.* **2002**, *50*, 6635–6642.
25. Brandon, D. L.; Bates, A. H.; Friedman, M. *J. Food Sci.* **2004**, *69*, C11–C15.
26. Ware, J. H.; Wan, X. S.; Newberne, P.; Kennedy, A. R. *Dig. Dis. Sci.* **1999**, *44*, 986–990.
27. Lichtenstein, G. R.; Deren, J. J.; Katz, S.; Lewis, J. D.; Kennedy, A. R.; Ware, J. H. *Dig. Dis. Sci.* **2008**, *53*, 175–180.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

28. Kennedy, A. R.; Kritchevsky, D.; Shen, W.-C. *Pharm. Res.* **2003**, *20*, 1908–1910.
29. Arbogast, S.; Smith, J.; Matuszczak, Y.; Hardin, B. J.; Moylan, J. S.; Smith, J. D.; Ware, J.; Kennedy, A. R.; Reid, M. B. *J. Appl. Physiol.* **2007**, *102*, 956–964.
30. Bawadi, H. A.; Antunes, T. M.; Shih, F.; Losso, J. N. *J. Agric. Food Chem.* **2004**, *52*, 4730–4736.
31. Yano, T.; Suzuki, K.; Asano, R. *Soy Protein Res. (Japan)* **2005**, *8*, 113–116.
32. Kennedy, A. R. The Status of Human Trials Utilizing Bowman-Birk Inhibitor Concentrate from Soybeans. In *Soy in Health and Disease Prevention*; Sugano, M., Ed.; CRC Press LLC: Boca Raton, FL, 2005; Chapter 12, pp 207−223.
33. Kennedy, A. R. Status of Current Human Trials Utilizing Bowman Birk Inhibitor Concentrate, Proceedings of the Soy & Health 2006, Dietetic Applications Symposium, Dusseldorf, Germany, October 12−13, 2006.
34. Kunitz, M. *Science* **1945**, *101*, 668–669.
35. Blow, D. M.; Janin, J.; Sweet, R. M. *Nature* **1974**, *249*, 54–57.
36. Sweet, R. M.; Wright, H. T.; Janin, J.; Chothia, C. H.; Blow, D. M. *Biochemistry* **1974**, *13*, 1599–1608.
37. Song, H. K.; Suh, S. W. *J. Mol. Biol.* **1998**, *275*, 347–363.
38. Roychaudhuri, R.; Sarath, G.; Zeece, M.; Markwell, J. *Arch. Biochem. Biophys.* **2003**, *412*, 20–26.
39. Tetenbaum, J.; Miller, L. M. *Biochemistry* **2001**, *40*, 12215–12219.
40. Singh, L.; Wilson, C. M.; Hadley, H. H. *Crop Sci.* **1969**, *9*, 489–491.
41. Hymowitz, T. *Crop Sci.* **1973**, *13*, 420–421.
42. Orf, J. H.; Hymowitz, T. *Theor. Crop Sci.* **1979**, *19*, 107–109.
43. Zhao, S. W.; Wang, H. *Soybean Genet. Newsl.* **1992**, *19*, 22–24.
44. Wang, K. J.; Kaizuma, N.; Takahata, Y.; Hatakeyama, S. *Breed Sci.* **1996**, *46*, 39–44.
45. Wang, J. J.; Takahata, Y.; Ito, K.; Zhao, Y. P.; Tsutsumi, K.; Kaizuma, N. *Breed. Sci.* **2001**, *51*, 185–190.
46. Wang, K. J.; Yamashita, T.; Watanabe, M.; Takahata, Y. *Genome* **2004**, *47*, 9–14.
47. Wang, K. J.; Takahata, Y.; Kono, Y.; Kaizuma, N. *Theor. Appl. Genet.* **2005**, *112*, 66–71.
48. Duranti, M.; Barbiroli, A.; Scarafoni, A.; Tedeschi, G.; Morazzoni, P. *Protein Expression Purif.* **2003**, *30*, 167–170.
49. Sugarawa, M.; Ito, D.; Yamamoto, K.; Akita, M.; Oguri, S.; Momonoki, Y. *Plant Prod. Sci.* **2007**, *10*, 314–321.
50. Shakiba, Y.; Mansouri, K.; Mostafaie, A. *Fitoterapia* **2007**, *78*, 587–589.
51. Wang, K. J.; Takahat, Y.; Kono, Y.; Kaizuma, N. *Theor. Appl. Genet.* **2008**, *117*, 565–573.
52. Brandon, D. L.; Bates, A. H. *J Agric. Food Chem.* **1988**, *36*, 1336–1341.
53. Kobayashi, H.; Yoshida, R.; Kanada, Y.; Fukuda, Y.; Yagyu, T.; Inagaki, K.; Kondo, T.; Kurita, N.; Suzuki, M.; Kanayama, N.; Terao, T. *J. Periodontal Res.* **2005**, *40*, 461–468.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

54. Kobayashi, H.; Yoshida, R.; Kanada, Y.; Fukuda, Y.; Yagyu, T.; Inagaki, K.; Kondo, T.; Kurita, N.; Yamada, Y.; Sado, T.; Kitanaka, T.; Suzuki, M.; Kanayama, N.; Terao, T. *Exp. Dermatol.* **2005**, *14*, 765–774.
55. Kobayashi, H.; Suzuki, M.; Kanayama, N.; Terao, T. *Clin. Exp. Metastasis* **2004**, *21*, 159–166.
56. Seiberg, M.; Shapiro, S.; Paine, C.; Conney, A. H.; Huang, M.-T. Topican Anticancer Compositions and Methods of Use Thereof. U.S. Patent 7,309,688 B2, December 18, 2007.
57. Garthoff, L. H.; Henderson, G. R.; Sager, A. O.; Sobotka, T. J.; Gaines, D. W.; O'Donnell, M. W., Jr.; Chi, R.; Chirtel, S. J.; Barton, C. N.; Brown, L. H.; Hines, F. A.; Solomon, T.; Turkleson, J.; Berry, D.; Dick, H.; Wilson, F.; Khan, M. A. *Food Chem. Toxicol.* **2002**, *40*, 501–16.
58. Garthoff, L. H.; Henderson, G. R.; Sager, A. O.; Sobotka, T. J.; O'Dell, R.; Thorpe, C. W.; Trotter, W. J.; Bruce, V. R.; Dallas, H. L.; Poelma, P. L.; Solomon, H. M.; Bier, J. W.; O'Donnell, M. W., Jr.; Chi, R. K.; Chirtel, S. J.; Barton, C. N.; Brown, L. H.; Frattali, V. P.; Khan, M. A. *Food Chem. Toxicol.* **2002**, *40*, 487–500.
59. Galvez, A. F.; Chen, N.; Macasieb, J.; de Lumen, B. O. *Cancer Res.* **2001**, *61*, 7473–7478.
60. Jeong, H. J.; Lam, Y.; de Lumen, B. O. *J. Agric. Food Chem.* **2002**, *50*, 5903–5908.
61. Jeong, J. H.; Jeong, J. B.; Kim, D. S.; de Lumen, B. O. *J. Agric. Food Chem.* **2007**, *55*, 632–637.
62. Jeong, H. J.; Jeong, J. B.; Kim, D. S.; Park, J. O.; Lee, J. B.; Kweon, D. H.; Chung, G. Y.; Seo, E. W.; de Lumen, B. O. *Cancer Lett.* **2007**, *255*, 42–48.
63. Silva-Sanchez, C.; Barba de la Rosa, A. P.; Leon-Galvan, M. F.; de Lumen, B. O.; de Leon Rodriguez, A.; Gonzalez de Mejia, E. *J. Agric. Food Chem.* **2008**, *56*, 1233–1240.
64. Jeong, J. H.; Park, J. H.; Lam, Y.; de Lumen, B. O. *J. Agric. Food Chem.* **2003**, *41*, 7901–7906.
65. de Mejia, E. G.; Vasconez, M.; de Lumen, B. O.; Nelson, R. *J. Agric. Food Chem.* **2004**, *52*, 5882–5887.
66. Hernandez-Ledesma, B.; Hsieh, C. C.; de Lumen, B. O. *Food Chem.* **2009**, *115*, 574–580.
67. Lam, Y.; Galvez, A.; de Lumen, B. O. *Nutr. Cancer* **2003**, *47*, 88–94.
68. de Mejia, E. G.; Dia, V. P. *Peptides* **2009**, *30*, 2388–2398.
69. Hernandez-Ledesma, B.; Hsieh, C. C.; de Lumen, B. O. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 803–808.
70. Gonzalez de Mejia, E.; Wang, W.; Dia, V. P. *Mol. Nutr. Food Res.* **2010**, *54*, 1–9.
71. Dia, V. P.; Torres-Diaz, S.; de Lumen, B. O.; Erdman, J. W.; Gonzalez de Mejia, E. *J. Agric. Food Chem.* **2009**, *57*, 1260–1266.
72. Wang, W.; Bringe, N. A.; Berhow, M. A.; Gonzalez de Mejia, E. *J. Agric. Food Chem.* **2008**, *56*, 4012–4020.
73. Liu, K. S. *Soybeans: Chemistry, Technology, and Utilization*; Aspen Publishers, Inc.: Gaithersburg, MD, 1997.

74. Utsumi, S.; Matsumura, Y.; Mori, T. In *Food Proteins and Their Applications*; Marcel Dekker, Inc.: New York, 1997; pp 257−291.
75. Thanh, S.; Shibasaki, K. *J. Agric. Food Chem.* **1979**, *27*, 805–809.
76. Maruyama, N.; Adachi, M.; Takahashi, K.; Yagasaki, K.; Kohno, M.; Takenaka, Y.; Okuda, E.; Nakagawa, S.; Mikami, B.; Utsumi, S. *Eur. J. Biochem.* **2001**, *268*, 3595–3604.
77. Maruyama, Y.; Maruyama, N.; Mikami, B.; Utsumi, S. *Acta Crystallogr.* **2004**, *60*, 289–297.
78. Maruyama, N.; Salleh, M. R. M.; Takahashi, K.; Yagasaki, K.; Goto, H.; Hontani, N.; Nakagawa, S.; Utsumi, S. *J. Amer. Oil Chem. Soc.* **2002**, *79*, 139–144.
79. Nagano, T.; Hirotsuka, M.; Mori, H.; Kohyama, K.; Nishinar, K. *J. Agric. Food Chem.* **1992**, *40*, 941–944.
80. Hou, H. J.; Chang, K. C. *J. Agric. Food Chem.* **2004**, *52*, 7931–7937.
81. Zuo, W. Y.; Chen, W. H.; Zhou, S. X. *World J. Gastroenterol.* **2005**, *11*, 5801–5806.
82. Shen, C. L.; Chen, W. H.; Zou, S. X. *J. Appl. Microbiol.* **2006**, *102*, 283–289.
83. Yang, B.; Ying, L. V.; Chen, Y.; Wang, J.; Tang, W.; Guo, S. *J. Agric. Food Chem.* **2008**, *56*, 7522–7527.
84. Ohinata, K.; Shun, A.; Yoshikawa, M. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 2618–2621.
85. Martinez-Villaluenga, C.; Bringe, N. A.; Berhow, M. A.; Gonzalez de Mejia, E. *J. Agric. Food Chem.* **2008**, *56*, 10533–10543.
86. Martinez-Villaluenga, C.; Dia, V. P.; Berhow, M.; Bringe, N. A.; Gonzalez de Mejia, E. *Mol. Nutr. Food Res.* **2009**, *53*, 1007–1018.
87. Mochizuki, Y.; Maebuchi, M.; Kohno, M.; Hirotsuka, M.; Wadahama, H.; Moriyama, T.; Kawada, T.; URA, R. Changes in lipid metabolism by soy β-conglycinin-derived peptides in HepG2 cells. *J. Agric. Food Chem.* **2009**, *57*, 1473–1480.
88. Durati, M.; Morazzoni, P. U.S. Patent 7,465,467 B2, December 16, 2008.
89. Park, J. H.; Jeong, H. J.; de Lumen, B. O. *J. Agric. Food Chem.* **2007**, *55*, 10703–10706.
90. Dia, V. P.; Berhow, M. A.; Gonzalez de Mejia, E. *J. Agric. Food Chem.* **2008**, *56*, 11707–11717.
91. De Meester, P.; Brick, P.; Lloyd, L. F.; Blow, D. M.; Onesti, S. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1998**, *54*, 589–597.
92. Wang, W.; Rupasinghe, S. G.; Schuler, M. A.; Gonzalez de Mejia, E. *J. Agric. Food Chem.* **2008**, *56*, 6267–6277.
93. Fu, C. J.; Jez, J. M.; Kerley, M. S.; Allee, G. L.; Krishnan, H. B. *J. Agric. Food Chem.* **2007**, *55*, 4014–4020.
94. Tsuruki, T.; Takahata, K.; Yoshikawa, M. *Peptides* **2005**, *26*, 707–711.
95. Mao, Y. F.; Lai, C.; Vogtentanz, G.; Schmidt, B.; Day, T.; Miller, J.; Brandon, D. L.; Chen, D. *Protein J.* **2005**, *24*, 275–282.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch009

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

Chapter 10

Changes of Functional Components and Antioxidative Activity in the Process of Fermentation of Soybeans

Junko Yamashita, Hua-Ming Chen, Takako Naganuma, Tomohisa Ogawa, and Koji Muramoto*

**Department of Biomolecular Sciences, Graduate School of Life Sciences,
Tohoku University, Sendai 981-8555, Japan
*muramoto@biochem.tohoku.ac.jp**

We examined changes of functional components and antioxidative activity of soybeans during the processes for producing a fermented soybean food, natto. Proteolytic hydrolysis of proteins was followed along with the changes in the contents of isoflavones, saponins, and phytic acid. The antioxidative activities were measured by radical scavenging, Fe^{2+} chelating, superoxide dismutase-like activity, and β-carotene-linoleic acid assay. Radical scavenging activity under hydrophilic conditions increased with protein hydrolysis during fermentation. At the same time, total isoflavones decreased. Total saponins and phytic acid were kept at similar levels. The analyses of functional components and antioxidative activities showed that antioxidative peptides generated during fermentation contributed to the total antioxidative capacity of natto.

Many proteins and their hydrolysates have been shown to have antioxidative activities against the peroxidation of lipids or fatty acids upon hydrolysis (*1–4*). Thus, the antioxidative activities of amino acids and peptides have been investigated to gain insight into the antioxidative mechanism of protein hydrolysates. Several amino acids, such as Tyr, Met, His, Lys, CySH and Trp, are generally accepted as antioxidants despite their pro-oxidative effects in some

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

cases (*5–7*). The antioxidative activities of some dipeptides were demonstrated in an oil system (*8*) and in a metal-catalyzed liposomal suspension system (*9*). The antioxidative activities of dipeptides on linoleic acid autoxidation were investigated to reveal that the peptides had higher antioxidative activities than the constituent amino acid mixtures (*10*).

The positional selectivity and rates of radical attack on peptides and proteins accounted for the presence of the radical stabilizing groups on side chains. Site-specific oxidation can also arise from the binding of metal ions or other initiating species at particular sites on peptides. Selective oxidative modification of amino acid residues has been observed with various peptides and proteins (*11*, *12*). The antioxidative activities of Trp and Tyr may be explained by the special capability of phenolic and indolic groups to serve as hydrogen donors. The phenoxyl and indoyl radicals are much more stable and have longer lifetimes than simple peroxy radicals, so any reverse reaction and the propagation of the radical-mediated peroxidizing chain reaction are inhibited. These characteristic properties of peptides can be attributed to their antioxidative activities.

Six antioxidative peptides were isolated and characterized from the proteolytic digest of a soybean protein (*13*). The peptides were composed of 5-16 amino acid residues, including hydrophobic amino acids, Val or Leu, at the N-terminal positions, and Pro, His, or Tyr in the sequences. On the basis of the smallest peptide, Leu-Leu-Pro-His-His (LLPHH), 28 structurally related peptides were synthesized, and their antioxidative activities against the peroxidation of linoleic acid were compared in an aqueous system. Pro-His-His (PHH) was found to be the most active among the peptides tested (*14*). Further study with 22 synthetic peptides containing His residues demonstrated that His-containing peptides can act as a metal-ion chelator, an active-oxygen quencher, and a hydroxyradical scavenger (*15*). The results obtained so far indicate that the overall antioxidative activities of peptides are attributed to the cooperative effects of these properties.

Antioxidative peptides showed synergistic effects with nonpeptidic antioxidants as observed in soybean protein hydrolysates (*2*). The magnitude of the effects, however, did not correlate with the antioxidative activities of the peptides (*14*). Two lines of tripeptide libraries were constructed based on an antioxidative peptide, one was a library of 108 peptides containing either His or Tyr residues, another was a library of 114 peptides related to Pro-His-His, and antioxidative properties and synergistic effect with phenolic antioxidants were examined (*16*). Tyr-His-Tyr showed the strongest synergistic effect with phenolic antioxidants. However, the structure-activity relationships of antioxidative peptides varied depending on the method used to measure antioxidative activity.

In this study, we analyzed the changes in the contents of functional components in soybeans during fermentation process to produce “natto”, a typical fermented soybean food. Comprehensive analyses of the components and the antioxidative activities under hydrophilic and hydrophobic conditions revealed that antioxidative peptides were produced and contributed significantly to the total antioxidative capacity of natto.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

Materials and Methods

Materials

Soybeans (*Glycine max*) (Kosuzu, small grains) were obtained from Japan Agricultural Co-operatives Miyagi. Trinitrobenzenesulfonic acid (TNBS), isoflavones (daidzein, glycitein, genistein, daidzin, glycitin, and genistin), β-carotene, sulfanilic acid, phytic acid sodium salt, 2, 2′-azinobis (3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), hydroxyl ammonium chloride, hypoxanthine, hydroxylamine *O*-sulfonic acid, and *N*-1-naphthylethylene-diamine were purchased from Nacalai Tesque (Kyoto, Japan). Xanthin oxidase (XOD) and linoleic acid were purchased from Wako Chemicals (Osaka, Japan). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), tetramethyl murexide ammonium salt (TMM), hexamethylene tetramine, and superoxide dismutase (SOD) (EC 1. 15. 1. 1) from bovine erythrocytes were purchased from Sigma Chemical (St. Louis, MO). All other reagents were the purest grade commercially available.

Preparation of Natto Sample

Bacillus subtilis (*natto*) was obtained from Tohoku Bioscience (Sendai, Japan). Eight hundred grams of soybeans were cleaned and soaked overnight until they doubled in weight. The soaked soybeans were steamed at 121°C for 30 min and then cooled down to 40°C. The steamed soybeans were inoculated with *B. subtilis* and incubated at 40°C for 20 h. Samples were taken from the fermented soybeans every 4 h. The fermented soybeans were further incubated at 4°C for another 20 h to complete maturation, and samples were taken at 10 h and 20 h. The samples, which had been taken at 0, 4, 8, 12, 16, 20, 30, and 40 h, were pulverized with a mortar and pestle, and lyophilized.

Each sample was processed for analysis as described below and stored at -20°C until use. Natto powder (NP): Natto was pulverized and lyophilized. Water extract (WE): Natto powder was extracted with distilled water. After centrifugation, the supernatant was lyophilized. Partially fractionated extract (PF): Natto powder was defatted with hexane and extracted with distilled water. Lyophilized extract was dissolved in distilled water (10 mg/ml) and applied to anion-exchange chromatography on a HiTrap SP XL column (5 ml) (Amersham Pharmacia Biotech, Sweden) equilibrated with distilled water. The column was washed with distilled water and the retarded fraction containing peptides was eluted with 2 M NH_4OH.

SDS-PAGE

Each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% acrylamide gels according to the method of Laemmli (*17*). The molecular markers used were bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myoglobin (18 kDa), and cytochrom c (13 kDa).

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

HPLC Analysis

Samples (10.0 mg) were dissolved in 1.0 ml of phosphate-buffered saline (PBS) and centrifuged at 17,400 g for 10 min. The sample solution (40 μl) was subjected to reversed-phase HPLC on a TSKgel ODS 120T column (4.6 × 250 mm) (Tosoh, Tokyo, Japan) using 0.1% trifluoroacetic acid (TFA) in water (Solvent A) and 80% acetonitrile containing 0.08% TFA (Solvent B) at a flow rate of 1.0 ml/min and 40°C. The gradient elution was performed from 0 to 50% Solvent B in 60 min, and from 50 to 100% Solvent B in next 10 min. The eluate was monitored at 220 nm.

Degree of Hydrolysis

The protein hydrolysis was determined as the degree of hydrolysis by the TNBS method (*18*) with a minor modification. Fifty microliters of samples (1.0 mg/ml) in 1% SDS were mixed with 400 μl of 0.2 M sodium phosphate buffer (pH 8.2). Four hundred microliters of 0.1 % TNBS solution (in distilled water) was added, and the mixture was vortexed and incubated at 50°C for 60 min. The reaction was terminated by the addition of 800 μl of 0.1 M HCl 30 min before the absorbance was read against water at 340 nm. The standard solutions were prepared with 50 mM L-Leucine in 1 % SDS. The absorbance values were the means of six individual determinations.

Isoflavones

Lyophilized sample (250.0 mg) was mixed with 0.1 M HCl (1.0 ml), acetonitrile (3.5 ml), and water (1.5 ml) in a 25-ml centrifuge tube. The tubes were vortexed for 1 min and shaken with a multi wrist shaker for 2 h at room temperature before centrifuging at 4500 rpm for 30 min. An aliquot (2.0 ml) of the supernatant was transferred to a test tube and dried by a centrifugal evaporator. Duplicate extracts were prepared from each sample. Dried samples were dissolved in 1.0 ml of 80% acetonitrile, vortexed and centrifuged (17,400 g, 10 min) prior to HPLC analysis (*19*). Reversed-phase HPLC was carried out on a Capcellpak AG120A column (4.6 × 250 mm) (Shiseido, Tokyo, Japan) using the mobile phase consisted of 1% acetic acid in water (Solvent A) and acetonitrile containing 1% acetic acid (Solvent B) at a flow rate of 1.0 ml/min and 40°C. The column was eluted using the following solvent gradient: 90-80% Solvent A in 20 min; 80-70% from 20 to 60 min; and 70-0% from 60 to 61 min.

Authentic standards of daidzein, glycitein, genistein, daidzin, glycitin, and genistin were dissolved in 80% aqueous methanol. The concentration of working solutions was determined by measuring of UV absorbance (*20*). Isoflavone content was expressed as μmol/g soybeans.

Group B Saponins

Lyophilized sample (75.0 mg) was extracted with 1.5 ml of 70% aqueous ethanol with shaking for 2.5 h at room temperature before centrifuging at 17,400 g

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

for 30 min. The extraction was repeated under the same conditions. The combined supernatant was placed in a glass tube and dried by a centrifugal evaporator (*21*). The residue was dissolved in 0.3 ml of 80% aqueous methanol and subjected to reversed-phase HPLC on a Capcellpak AG120A column (4.6 × 250 mm) at a flow rate of 1.0 ml/min and 40°C using the mobile phase of 0.1% TFA in water (Solvent A) and 80% acetonitrile containing 0.08% TFA (Solvent B). The column was eluted using the following solvent gradient: 40-45% Solvent B in 10 min; 45-50% from 10 to 70 min; and 50-100% from 70 to 71 min. The eluate was monitored at 220 nm. Group B saponins were isolated from soyasaponins (Fuji Oil, Osaka, Japan) and used as standards.

Phytic Acid

Lyophilized sample was defatted by extracting twice with 5 volumes of hexane and dried in a hood over night. Defatted sample (75.0 mg) was mixed with 0.66 M HCl (1.5 ml), vortexed for 1 min and shaken for 2 h at room temperature before centrifuging at 17,400 g for 30 min (*22*). The supernatant (1.0 ml) was diluted to 5.0 ml with distilled water, and loaded onto a column of Dowex 1×4 anion exchange resin (100-200 mesh) (1 g). Before use, the column was washed with 20 ml of 10% NaCl and then with 30 ml of distilled water to saturate the resin with chloride ions. Inorganic phosphorus was eluted with 15 ml of 0.1 M NaCl, and bound phytic acid was eluted with 20 ml of 0.7 M NaCl. The first 3.0 ml was collected for analysis. One hundred microliters of the modified Wage reagent (0.3% $FeCl_3$ and 3% sulfosalicylic acid in distilled water) was added to 3.0 ml of sample solution or standard solutions, and mixed for 5 sec. Absorbance was measured at 500 nm. A series of standard solutions (6.25-100 µg/ml phytic acid sodium salt in distilled water) were used for a calibration curve.

ABTS Radical Scavenging Activity

The ABTS radical scavenging activity was measured based on the ability of samples to scavenge the long-life $ABTS^+$ radical cation (*23*, *24*). Lyophilized sample (10.0 mg) was dissolved in 1.0 ml of PBS and centrifuged at 17,400 g for 15 min. The supernatants were collected and adequately diluted with PBS. Metmyoglobin and ABTS were mixed to final concentrations of 2.5 µM and 150 µM, respectively. After pre-incubating at 40°C, $ABTS^+$ radical cation was generated by the addition of H_2O_2 to be 150 µM for 18 min. The mixture (100 µl) was added to 20 µl of test samples in a titer plate. The plate was shaken for 30 sec and the absorbance at 630 nm was measured by a titer plate reader (Model 680 Microplate Reader, Bio-Rad). A series of standard solutions of Trolox (15.6-250 µM) were used for a calibration curve. The results were expressed as the Trolox equivalent antioxidant capacity (TEAC) value.

DPPH Radical Scavenging Activity

Lyophilized sample (10.0 mg) was suspended in 1.0 ml of methanol overnight and centrifuged at 17,400 g for 15 min. The supernatant was collected and diluted with methanol appropriately. Fifty microliters of samples in a titer plate and 0.15 mM DPPH (100 µl) in methanol were mixed (*24*, *25*). The plate was shaken for 30 sec and measured the absorbance at 510 nm by a titer plate reader. The plate was kept in dark at 4°C and the absorbance was measured every 30 min. A series of standard solutions of Trolox (0.3-25 µM) were used for a calibration curve. The results were expressed as the TEAC.

Fe^{2+}-Chelating Activity

Fe^{2+}-Chelating activity was measured by the TMM-Fe^{2+} complex method (*26*) with a minor modification. Lyophilized sample (5.0 mg) was suspended in 1.0 ml of 10 mM hexamethylenetetramine (hexamine) buffer (pH 5.0) containing 10 mM KCl for 1 h and centrifuged at 17,400 g for 15 min. The supernatant was collected and diluted with hexamine buffer for measuring. Eighty microliters of the sample solution was added to 80 µl of 10 mM hexamine buffer containing 10 mM KCl and 3 mM $FeSO_4$, and then 15 µl of 0.5 mM TMM was added. Absorbance at 490 and 540 nm was measured. When TMM was added to 0.19-3 mM $FeSO_4$ solution for the standard curve, the absorbance ratio (A_{480}/A_{540}) increased linearly with Fe^{2+} (TMM-Fe^{2+} complex) concentration.

β-Carotene-Linoleic Acid Method

The method was based on the decoloration of β-carotene by the peroxides generated during the oxidation of linoleic acid at elevated temperature (*27*). In brief, β-carotene (0.1 mg) was dissolved in 100 µl of $CHCl_3$ in a 50-ml round-bottom flask, to which 80 µl of linoleic acid (100 mg/ml) and 200 µl of Tween 20 (200 mg/ml) were added. $CHCl_3$ was removed with N_2 gas. Distilled water (18 ml) and 0.2 M sodium phosphate buffer (pH 6.8) containing 150 mM NaCl (2 ml) were added, and the flask was shaken vigorously until all materials dissolved. This test mixture was prepared freshly and used immediately. Fifty microliters of sample solution and 400 µl of the mixture were mixed and heated at 50°C. After 45 min, absorbance was measured at 492 nm. The results were expressed as the antioxidant potency of known amounts of Trolox.

Superoxide Dismutase Activity

Superoxide dismutase (SOD)-like activity was determined by following the inhibition of nitrite formation from hydroxyl ammonium chloride in the presence of superoxide generators (*28*). The sample (50 µl), Reagent A (150µl substrate reagent: aqueous solution, pH 7.0, to final concentration of 1 mM hydroxyl ammonium chloride, 0.1 mM hypoxanthine, 0.1 g/ml hydroxylamine *O*-sulfonic acid), buffer (100 µl, 13 mM KH_2PO_4, 7 mM sodium borate, 0.1 mM EDTA-Na, pH 8.2) and distilled water (1.0 ml) were mixed. The reaction was started by adding 0.1 ml of enzyme solution to final concentration of 1.25 mU/ml XOD. The mixture was incubated for 30 min at 37°C without shaking and received Reagent B (1.0 ml coloring reagent to final concentration of 2 mM sulfanilic acid, 20 µM N-1-naphthylethylenediamine, and 16.7% acetic acid). The final mixture

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

was allowed to stand for 20 min at room temperature, and the absorbance at 550 nm was measured. A series of standard solutions (0.12-30 U/ml SOD in distilled water) were used for a calibration curve.

Results

Natto samples were analyzed by SDS-PAGE and the TNBS method to assess protein hydrolysis (Fig. 1). The proteins in the samples stayed intact until 8-h incubation. The sample of 12-h suddenly showed an extensive hydrolysis of the proteins, indicating that *B. subtilis* actively grew by 12 h and vigorously secreted proteases after 8-h incubation. The hydrolysis continued moderately after 12 h. Typical peptide profiling of natto is show in Figure 2. Soaked soybeans gave few small peaks as expected. After steaming and fermentation, natto showed a number of peptide peaks. Most of them eluted with low concentration of acetonitrile, indicating the presence of small peptides.

Isoflavone contents in the samples of different fermentation periods were determined by reversed-phase HPLC (Fig. 3). Among the isoflavones, genistin content was the highest, followed by daidzin and glycitein that agreed with the previous report (*19*). The total isoflavone content increased by steaming process and then decreased during fermentation process. The fermentation process decreased the isoflavone contents. DDMP-conjugated group B saponins, αg and βg, and their non-DDMP saponins, Ba(V) and Bb(I) were analyzed by reversed-phase HPLC (Fig. 4). Among the group B saponins, the βg saponin content was the highest, followed by Bb, βg and αg as previously reported (*21*). The total saponin content decreased gradually during fermentation. The phytic acid content (~1.3%) was consistent in the literature (*29*) (Fig. 5). The steaming and fermentation process did not affect the phytic acid content in soybeans.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

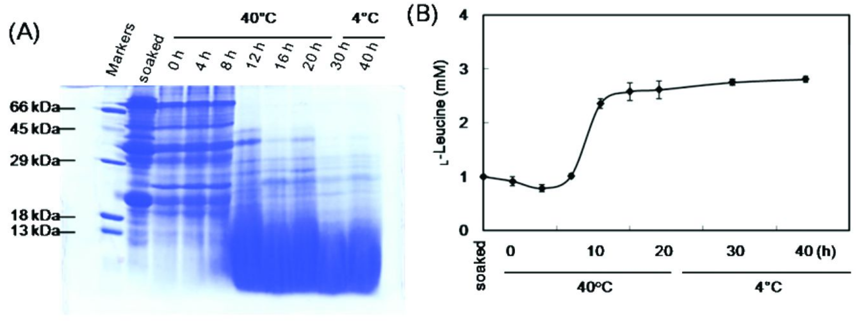


Figure 1. Protein hydrolysis analyzed by SDS-PAGE and TNBS method. (A) SDS-PAGE, (B) TNBS method.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

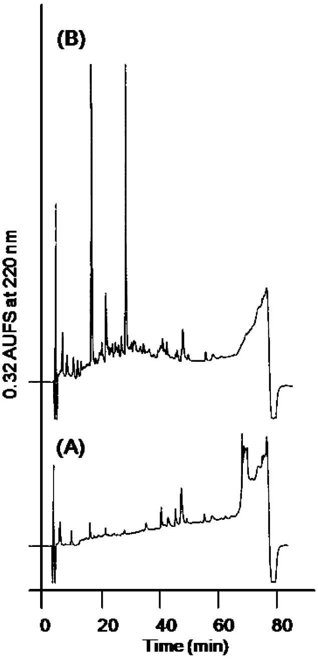


Figure 2. Peptide maps of natto analyzed by reversed-phase (RP-HPLC). (A) Soaked soybeans, (B) matured natto (treated for 40 h). RP-HPLC was carried out on a TSKgel ODS 120T column (4.6 × 250 mm) using 0.1% trifluoroacetic acid (TFA) in water (Solvent A) and 80% acetonitrile containing 0.08% TFA (Solvent B) at a flow rate of 1.0 ml/min and 40°C. The gradient elution was performed from 0 to 50% Solvent B in 60 min, and from 50 to 100% Solvent B in next 10 min. The eluate was monitored at 220 nm.

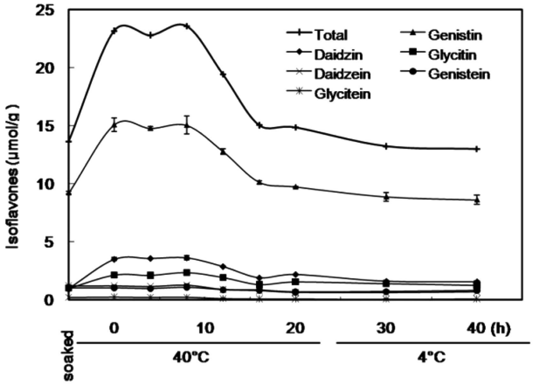


Figure 3. Changes of isoflavone contents during fermentation of soybeans. Isoflavone contents were measured as described in the text. Total isoflavone content of six isoflavones contents is shown as a heavy line.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

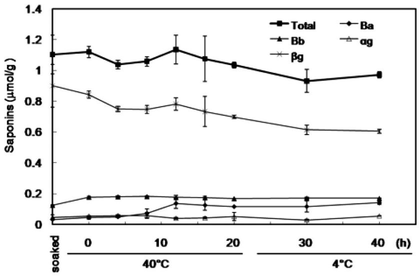


Figure 4. Changes of saponin contents during fermentation of soybeans. Saponin contents were measured as described in the text.

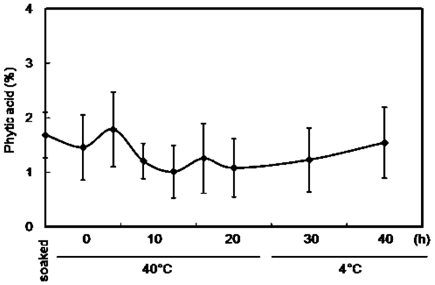


Figure 5. Changes of phytic acid content during fermentation of soybeans. Phytic acid contents (w/W) were measured as described in the text.

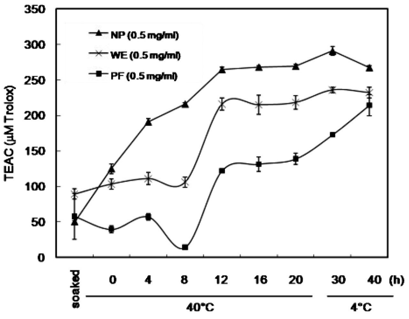


Figure 6. ABTS radical scavenging activity. The activity of each sample (0.5 mg/ml) is expressed as TEAC.

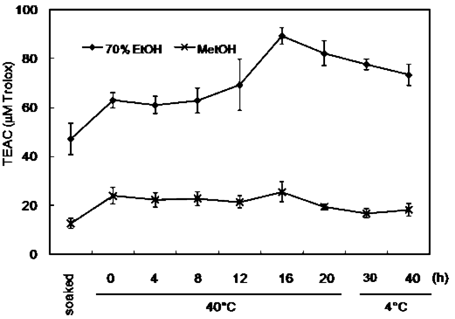


Figure 7. DPPH radical scavenging activity. The activity of each sample (10 mg/ml) is expressed as TEAC.

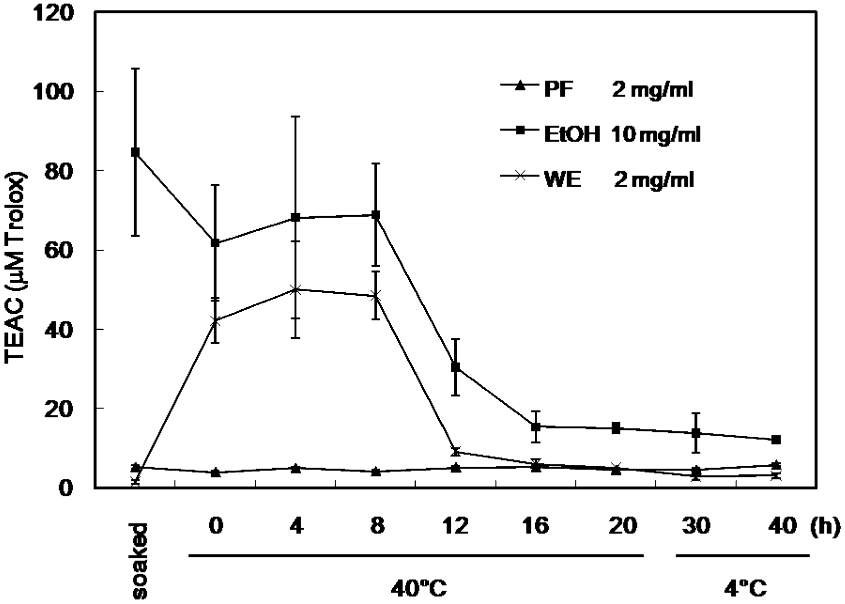


Figure 8. Antioxidant activity measured by the β-carotene-linoleic acid method. The activity against the peroxidation of linoleic acid is expressed as TEAC.

The ABTS radical-scavenging activity of natto powder (NP) gradually increased and leveled off at 12 h. On the other hand, the water extract (WE) and the partially fractionated extract (PF) showed no increase of TEAC until 8 h, and then rapidly increased until 12 h (Fig. 6). The results of the measurement of DPPH radical scavenging activity were also expressed as TEAC (Fig. 7). The lyophilized samples were extracted with 70% aqueous ethanol or 100% methanol. The extracts with 70% ethanol showed higher activity than that with 100% methanol, indicating that some antioxidants were more soluble in 70% ethanol than in methanol. The activity of the extracts with 70% ethanol sharply increased

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

at soaking and 16 h, and then rather decreased during maturation. Meanwhile, the methanol extract kept its activity during fermentation and maturation.

The antioxidative activity measured by the β-carotene-linoleic acid method is shown in Figure 8. For comparison with other measurements, the results are expressed as TEAC. The ethanol extracts showed the highest activity in the three samples. The activity decreased after 12-h fermentation. The water extract of soaked soybeans possessed lipoxygenase activity, which acted as a pro-oxidant for linoleic acid to lead the bleaching of β-carotene. The activity, except for the soaked one, was decreased after 12-h fermentation as the ethanol extracts. The partially fractionated extracts (PF) obtained by anion-exchange chromatography showed only weak antioxidative activities.

Ferrous ions (Fe^{2+}) are the most powerful pro-oxidant among various species of metal ions. Soybeans contain various metal chelator, e.g., flavones (*30*) and phytic acid (*31*). Fe^{2+}-binding activity in fermented soybeans was measured (Fig. 9). Dose-dependent Fe^{2+}-binding activities were observed with all samples tested. The activity was stable until 8-h fermentation, and then decreased by about 30% to level off. SOD-like activity was measured to determine the superoxide scavenging activity (Fig. 10). The activity was high with soaked soybeans and diminished by steaming. It gradually increased during fermentation.

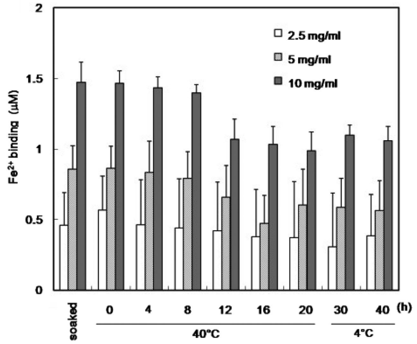


Figure 9. Fe^{2+} Chelating activity. The activity of each sample (2.5, 5, 10 mg/ml) was measured as described in the text.

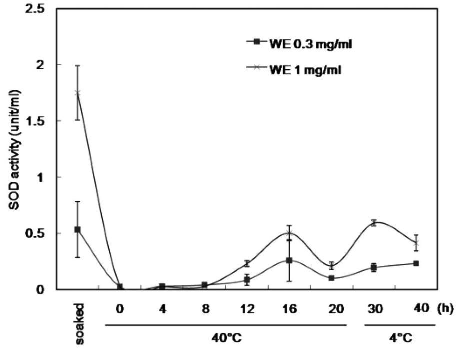


Figure 10. SOD-like activity. The activity of water extracts (WE) of natto were measured as described in the text.

Discussion

Fermented soybean foods have been shown to have a range of bioactive peptides with reduced allergenic reactivity due to proteolytic digestion of constituted proteins (*32*, *33*). Natto is a typical fermented soybean food which is prepared by several processes including soaking, steaming, fermentation with *B. subtilis* (*natto*), and maturation. Steaming and fermentation are critical processes that affect the properties and functional components of soybeans (*34*, *35*). In this study, the steaming increased total isoflavones about 80% (Fig. 3). The process might increase the extractability of isoflavones from soybean tissues. The deglycosylation of isoflavones to their aglycones could not be observed during fermentation, being in agreement with the lack of β-glucosidases in *B. subtilis* (*natto*) (*36*). The decrease of total isoflavones might be caused by conversion of isoflavones to other forms except aglycones. Meanwhile, only some DDMP conjugated saponins, αg and βg, converted to non-DDMP conjugated saponins, Ba and Bb, respectively, during fermentation.

Peroxyl radical scavenging assay was performed both in hydrophilic and hydrophobic conditions. ABTS and DPPH radical scavenging assay are usually classified as single electron transfer reactions; these two radicals may be neutralized either by direct reduction via electron transfers or by radical quenching via hydrogen atom transfer. One of the major factors affecting the activity of antioxidants that scavenge free radicals is their partitioning behavior in lipids and water (*37*). ABTS and DPPH radical were adapted to determine the hydrophilic and hydrophobic antioxidative capacity, respectively. ABTS radical scavenging activity was well correlated with protein hydrolysis, which could generate antioxidative peptides (Fig. 6). The water extracts (WE) had higher activities than the partially fractionated extracts (PF), which contained peptides retarded by ion exchange chromatography. The higher activities of WE might be attributed to the presence of phenolic compounds (*35*). WE did not contain isoflavones or saponins (data not shown).

Hydrophobic antioxidative activity and lipid peroxidation inhibitory activity were measured by the DPPH radical scavenging method and the β-carotene-linoleic acid method, respectively. The TEAC obtained by the DPPH method maintained high levels during fermentation, and did not correspond to the changes of functional components in soybeans (Fig. 7). On the other hand, the TEAC measured by the β-carotene-linoleic acid method reflected the decrease of isoflavone contents (Fig. 8). PF showed low TEAC (<6 μM TEAC, in 2 mg/ml sample) in the β-carotene-linoleic acid method, suggesting that the peptides alone were less effective against the inhibition of lipid peroxidation.

Fe^{2+} chelating activity decreased during 8-12-h fermentation (Fig. 9). The decrease corresponded to the decrease of isoflavone contents and protein hydrolysis, though isoflavones showed only weak metal-ion chelating activity (*38*). SOD have been isolated from soybeans (*39*, *40*). This is not surprising if the steaming process inactivated SOD. *B. subtilis* possesses SOD in both vegetative cells and spores (*41*). The increase of SOD-like activity during fermentation might be attributed to endogenous SOD of the bacterium and metabolized components (*42*).

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

In summary, the contents of functional components and the antioxidative activity of soybeans changed upon the processes of steaming and fermentation when producing natto. The peptides generated by the fermentation of soybeans may significantly contribute to the antioxidative activity of natto. However, the antioxidative activity is dependent not only on the peptides but also on other components, such as phenolic components. The synergistic effects of peptides and other components can explain the apparent high antioxidative activity of natto.

Acknowledgments

This work was supported by a grant from The Salt Science Foundation (no. 0825 to K.M.).

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

References

1. Bishov, S. J.; Henick, A. S. Antioxidant effect of protein hydrolyzates in a freeze-dried model system. *J. Food Sci.* **1972**, *37*, 873–875.
2. Yamaguchi, N.; Yokoo, Y.; Fujimaki, M. Studies on antioxidative activities of amino compounds on fats and oils. Part III. Antioxidative activities of soybean protein hydrolyzates and synergistic effect of hydrolyzate on tocopherol. *Nippon Shokuhin Kogyo Gakkaishi* **1975**, *22*, 431–435.
3. Yee, J. J.; Shipe, W. F.; Kinsella, J. E. Antioxidant effects of soy protein hydrolysates on copper-catalyzed methyl linoleate oxidation. *J. Food Sci.* **1980**, *45*, 1082–1083.
4. Tsuge, N.; Eikawa, Y.; Nomura, Y.; Yamamoto, M.; Sugisawa, K. Antioxidative activity of peptides prepared by enzymatic hydrolysis of egg-white albumin. *Nippon Nogei Kagaku Kaishi* **1991**, *65*, 1635–1641.
5. Marcuse, R. Antioxidative effect of amino-acids. *Nature* **1960**, *186*, 886–887.
6. Karel, M.; Tannenbaum, S. R.; Wallace, D. H.; Maloney, H. Autoxidation of methyl linoleate in freeze-dried model systems. III. Effects of added amino acids. *J. Food Sci.* **1966**, *31*, 892–896.
7. Yamaguchi, N. Studies on antioxidative activities of amino compounds on fats and oils. Part I. Oxidation of methionine during course of autoxidation of linoleic acid. *Nippon Shokuhin Kogyo Gakkaishi* **1971**, *18*, 313–318.
8. Kawashima, K.; Itoh, H.; Miyoshi, M.; Chibata, I. Antioxidant properties of branched-chain amino acid derivatives. *Chem. Pharm. Bull.* **1979**, *27*, 1912–1916.
9. Yamashoji, S.; Kajimoto, G. Antioxidant effect of Gly-Gly-His on Cu(II)-catalyzed autoxidation and photosensitized oxidation of lipids. *Agric. Biol. Chem.* **1980**, *44*, 2735–2736.
10. Yamaguchi, N.; Yokoo, Y.; Fujimaki, M. Studies on antioxidative activities of amino compounds on fats and oils. Part II. Antioxidative activities of dipeptides and their synergistic effects of tocopherol. *Nippon Shokuhin Kogyo Gakkaishi* **1975**, *22*, 431–435.

11. Davies, M. J. The oxidative environment and protein damage. *Biochim. Biophys. Acta* **2005**, *1703*, 93–109.
12. Hawkins, C. L.; Morgan, P. E.; Davies, M. J. Quantification of protein modification by oxidants. *Free Rad. Biol. Med.* **2009**, *46*, 965–988.
13. Chen, H.-M.; Muramoto, K.; Yamauchi, F. Structural analysis of antioxidative peptides from soybean β conglycinin. *J. Agric. Food Chem.* **1995**, *43*, 574–578.
14. Chen, H.-M.; Muramoto, K.; Yamauchi, F.; Nokihara, K. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *J. Agric. Food Chem.* **1996**, *44*, 2619–2623.
15. Chen, H.-M.; Muramoto, K.; Yamauchi, F.; Fujimoto, K.; Nokihara, K. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *J. Agric. Food Chem.* **1998**, *46*, 49–53.
16. Saito, K; Jin, D. H.; Ogawa, T.; Muramoto, K.; Hatakeyama, E.; Yasuhara, T.; Nokihara, K. Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. *J. Agric. Food. Chem.* **2003**, *51*, 3668–3674.
17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
18. Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobensenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262.
19. Lee, J. H.; Renita, M.; Fioritto, R. J.; Martin, S. K.; Schwartz, S. J.; Vodovotz, Y. Isoflavone characterization and antioxidant activity of Ohio soybeans. *J. Agric. Food Chem.* **2004**, *52*, 2647–2651.
20. Murphy, P. A.; Barua, K.; Hauck, C. C. Solvent extraction selection in the determination of isoflavones in soy foods. *J. Chromatogr., B* **2002**, *777*, 129–138.
21. Hu, J.; Lee, S.; Hendrich, S.; Murphy, P. Quantification of the group B soyasaponins by high-performance liquid chromatography. *J. Agric. Food Chem.* **2002**, *50*, 2587–2594.
22. Fruhbeck, G.; Alonso, R.; Marzo, F.; Santidrian, S. A modified method for the indirect quantitative analysis of phytate in foodstuffs. *Anal. Biochem.* **1995**, *225*, 206–212.
23. Miller, N. J.; Rice-Evans, C. A. Spectrophotometric determination of antioxidant activity. *Redox Rep.* **1996**, *2*, 161–171.
24. Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.
25. Ozcelik, B.; Lee, J. H.; Min, D. B. Effect of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. *Food Chem. Toxicol.* **2003**, *68*, 487–490.
26. Shimada, K.; Fujikawa, K.; Yahara, K.; Nakamura, T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* **1992**, *40*, 945–948.
27. Miller, H. E. A simplified method for the evaluation of antioxidants. *J. Am. Oil Chem. Soc.* **1971**, *48*, 91–95.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

28. Oyanagi, Y. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal. Biochem.* **1984**, *142*, 290–296.
29. Latta, M.; Eakin, M. A simple and rapid colorimetric method for phytate determination. *J. Agric. Food Chem.* **1980**, *28*, 1313–1315.
30. Mira, L.; Tereza Fernandez, M.; Santos, M.; Rocha, R.; Helena Florencio, M.; Jennings, K. R. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radical Res.* **2002**, *36*, 1199–1208.
31. Graf, E.; Eaton, J. W. Antioxidant functions of phytic acid. *Free Radical Biol. Med.* **1990**, *8*, 61–69.
32. Gibbs, B. F.; Zougman, A.; Masse, R.; Mulligan, C. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Res. Int.* **2004**, *37*, 123–131.
33. Frias, J.; Song, Y. S.; Martinez-Villaluenga, C.; De Mejia, E. G.; Vidal-Valverde, C. Immunoreactivity and amino acid content of fermented soybean products. *J. Agric. Food Chem.* **2008**, *56*, 99–105.
34. Fernandez-Orozco, R.; Frias, J.; Munoz, R.; Zielinski, H.; Piskula, M. K.; Kozlowska, H.; Vidal-Valverde, C. Fermentation as a bio-process to obtain functional soybean flours. *J. Agric. Food Chem.* **2007**, *55*, 8972–8979.
35. Xu, B.; Chang, S. K. C. Total phenolics, phenolic acids, isoflavones, and anthocyanins and antioxidant properties of yellow and black soybeans as affected by thermal processing. *J. Agric. Food Chem.* **2008**, *56*, 7165–7175.
36. Toda, T.; Uesugi, T.; Hirai, K.; Nukaya, H.; Tsuji, K.; Ishida, H. New 6-O-acyl isoflavone glycosides from soybeans fermented with *Bacillus subtilis* (*natto*). I. 6-O-succinylated isoflavone glycosides and their preventive effects on bone loss in ovariectomized rats fed a calcium-deficient diet. *Biol. Pharm. Bull.* **1999**, *22*, 1193–201.
37. Decker, E. A.; Warner, K.; Richards, M. P.; Shahidi, E. Measuring antioxidant effectiveness in food. *J. Agric. Food Chem.* **2005**, *53*, 4303–4310.
38. Mira, L.; Tereza-Fernandez, M.; Santos, M.; Rocha, R.; Helena-Florencio, M.; Jennings, K. R. Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radical Res.* **2002**, *36*, 1199–1208.
39. Crowell, D. N.; Amasino, R. M. Nucleotide sequence of an iron superoxide dismutase complementary. *Plant Physiol.* **1991**, *96*, 1393–1394.
40. Arahira, M.; Nong, V. H.; Kadokura, K.; Kimura, K.; Udaka, K.; Fukazawa, C. Molecular cloning and expression patterns of Cu/Zn-superoxide dismutases in developing soybean seeds. *Biosci,. Biotechnol., Biochem.* **1998**, *62*, 1018–1021.
41. Inaoka, T.; Matsumura, Y.; Tsuchido, T. Molecular cloning and nucleotide sequence of the superoxide dismutase gene and characterization of its product from *Bacillus subtilis*. *J. Bacteriol.* **1998**, *180*, 3697–703.
42. Iwai, K.; Nakaya, N.; Kawasaki, Y.; Matsue, H. Inhibitory effect of natto, a kind of fermented soybeans, on LDL oxidation *in vitro*. *J. Agric. Food Chem.* **2002**, *50*, 3592–3596.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch010

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Chapter 11

Thermal Effects on the Conversion of Isoflavones in Soybean

Hsi-Mei Lai* **and Pei-Yin Lin**

Department of Agricultural Chemistry, National Taiwan University No. 1,
Roosevelt Rd., Sec. 4, Taipei 10617, Taiwan
***hmlai@ntu.edu.tw**

Soybeans and soy-based products are getting popular in the world due to their nutritional and health-promoting benefits for human beings. The most human health related compounds in soys are isoflavones. There are twelve isoflavone conjugates existed in soybeans with different bioavailabilities and aglycones are reported to be the most effective one. The isoflavone profiles of soybeans can be changed by many factors, including the varieties of seed, pretreatments and thermal processing conditions. In this study, the effects of variety, pretreatment and thermal treatment on isoflavone profile of soybeans were evaluated. Two soybeans (KS1 and KS8) and two black soybeans (TN3 and TN6) grown domestically were soaked or germinated and followed by moist-thermal (121 °C up to 60 min) or dry-thermal (195 °C up to 30 min) treatments. The soaking and germination processes significantly increased the total amounts of isoflavones for 9-85%, depending on the soybean variety. During thermal processing, in general, malonylglucosides were significantly lost, corresponding with the increases of acetylglucosides and glucosides at early heating stage. Higher conversion ratio was found in dry-thermal process than in moist-thermal process. Aglycones could be effectively converted in matured soybeans after short dry-thermal treatment (10-20 min). For matured soybeans, the thermal processing is recommended for the conversion of aglycone conjugates from malonylglucosides, acetylglucosides, and glucosides. For soaked and germinated soybeans, dry thermal processing is not suggested due to its significant loss of total isoflavones and

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

insignificant increase of aglycone conjugates after long heating time.

Introduction

Soybeans and soy-based products are consumed daily by Asian people for centuries because of rich sources of proteins, unsaturated fatty acids, dietary fibers and phytochemicals. In the last decades, soy foods are widely promoted and consumed in the whole world due to their reported nutritional and health-promoting benefits. More and more studies have shown soy or its phytochemicals, especially, isoflavones, as effective cancer-preventive agents for several hormone-related diseases (*1*, *2*). Soybean isoflavones had been evidenced to be beneficial for the releasing menopausal symptoms (*3*) and preventing from osteoporosis of menopausal women (*4–6*) by stimulating bone formation and suppressing bone resorption (*7*).

Isoflavones are a subclass of flavonoids and also called phytoestrogen compounds due to their structural similarity with human hormone estradiol. There are three types of isoflavones in soybean, and each type exists in four different chemical forms, which include aglycoside conjugates (daidzein, genistein, and glycitein) and their β-glucoside conjugates: glucosides (daidzin, genistin and glycitin), malonylglycosides (6”-*O*-malonyldaidzin, 6”-*O*-malonylgenistin and 6”-*O*-malonylglycitin), and acetylglycosides (6”-*O*-acetyldaidzin, 6”-*O*-acetylgenistin and 6”-*O*-acetylglycitin). The bioavailability and bioactivity of isoflavones are affected by their chemical structures. It appears that aglycones of daidzein and genistein are more bioavailable than their conjugated forms in humans (*8*, *9*). The various bioavailabilities were also investigated among isoflavone families, as daidzein was found to be more bioavailable than genistein in adult woman (*10*). These observations indicated that deglycosylation of soy isoflavones achieved by applying optimal pretreatment or processing may significantly beneficial for human consumption.

The total content of isoflavones in unprocessed soybeans is dependent on the varieties with the ranges of 1.2 to 4.2 mg/g wet basis reported by Wang and Murphy (*11*, *12*) and 1.8 to 4.3 mg/g dry basis reported by Lin and Lai (*13*). The profiles of isoflavone isomers were similar among varieties, in which the malonylglycosides are the major components (56-76%) while the aglycones are trace (*13*). During processing, the content and profile of isoflavone isomers of the soybeans were variously changed depending on the types and degrees of processing. Soybean sprouts, the germinated soybeans, were reported contained high amounts of total isoflavones and aglycones, which were resulted from the bioconversion of seeds during germination. Lin and Lai (*13*) reported that the total isoflavones and aglycones content increased 28.6% and 19.5 times for a soybean (KS1) after 1-day germination.

Thermal processing is the most extensively used method of food preparation and preservation to destroy microorganisms thereby extending its shelf-life. The precooked soybean or soybean flour could be applied in many food items, such as instant drink mixes, desserts and snacks, for developing on the isoflavone

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

enriched functional soy-based foods. Vaidya et al. (*14*) indicated the differences in molecular structure between malonylgenistin and malonyldaidzin didn't affect their conversion rates of malonyl isomers to β-glucosides, but did affect the degradation rates of malonyl isomers while pH and heat increased. Chien et al. (*15*) indicated that the moist heating was more susceptible to conversion and degradation of isoflavones than dry heating when pure genistein and its conjugates in methanol were tested at 100, 150 and 200 °C. In soy milk, the genistein showed higher stability to heat treatment than daidzein and glycitein (*16*). Coward et al. (*1*) indicated that the toasted soy flour contained large amounts of 6"-*O*-acetyl-*β*-glucoside conjugates, formed by heat inducing decarboxylation of the malonate group to acetate. Converting 6"-*O*-malonylgenistin to 6"-*O*-acetyl-genistin during drying process and converting to genistin during hot water extraction were also reported by Barnes et al. (*17*).

More and more soy containing food products are continuously demanded because the beneficial effects of bioactive compounds in soybeans. The raw or precooked soybeans or soy flour could be used for several soy-based products. Thus, the conversion among various isoflavones, as affected by different combinations of pretreatments and thermal treatments, has to be investigated. The objective of this study was to evaluate the moist and dry thermal effects on the conjugated isoflavones with regard to interconversions in soybeans and black soybeans.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Materials and Methods

Sample Preparations

Two soybeans (*Glycine max* L.) (KS1 and KS8) and two black soybeans (TN3 and TN6) were bred and provided from Kaohsiung and Tainan District Agricultural Research and Extension Stations in Taiwan, respectively. Soybeans were pretreated with soaking and germination, followed by thermal treatments either by autoclave steaming (moist heating) or oven roasting (dry heating). For soaked soybeans, the matured seeds were soaked in tap water at 35 °C for 5 hr and then water was drained out. For germinated soybeans, the matured seeds were prepared as previous (*13*), which the soybeans and black soybeans were germinated for 1 day with 3-6 mm and 4 days with 3-6 cm length of sprouts in an incubator (GTH-150-20-CP-AR, Giant Force Co., Ltd., Taiwan) at 25 °C, 75% RH. The soaked and germinated soybeans were immediately frozen overnight at -30 °C and dried by freeze-dryer (FD-1240, PANCHUM Scientific Corp., Taiwan), or followed by the thermal treatments. The pretreated soybeans and black soybeans used for this study were shown in Figure 1.

Thermal Treatments of Soybeans

The pretreated soybeans, soaked and germinated, were followed by thermal treatment which was either steamed at 121 °C in an autoclave (CL-32L, ALP Co. Ltd., Japan) for 10 to 60 min or roasted at 195 °C in an oven (Chung Pu Baking Machinery Co. Ltd., Taichung, Taiwan) for 10, 20 and 30 min. After steaming, the samples were lyophilized as described above, ground and stored in a desiccator until use. The roasted soybeans were ground immediately after roasting and stored in a desiccator, except for the 10 min-roasted soybeans. The short roasting time could not efficiently remove water from soybeans and caused the difficulty in grounding samples so that the roasted soybeans were lyophilized before grinding.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

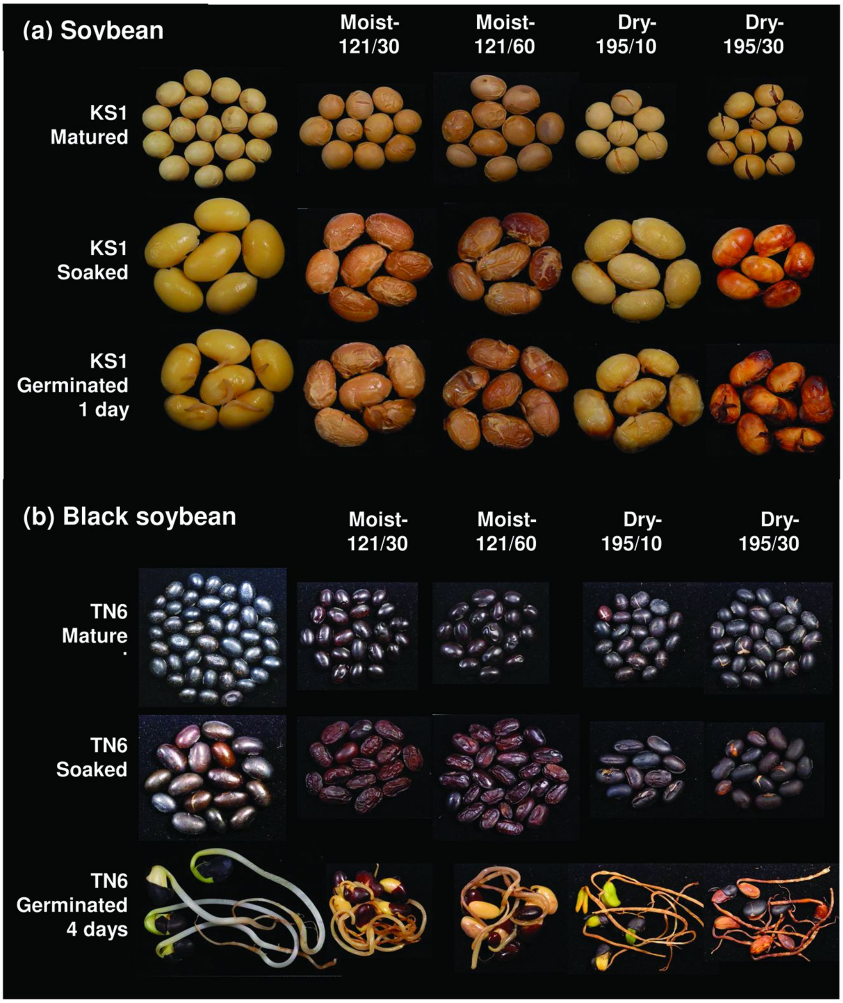

(a) Soybean
Moist-121/30
Moist-121/60
Dry-195/10
Dry-195/30
KS1 Matured
KS1 Soaked
KS1 Germinated 1 day
(b) Black soybean
Moist-121/30
Moist-121/60
Dry-195/10
Dry-195/30
TN6 Mature
TN6 Soaked
TN6 Germinated 4 days

Figure 1. The pretreated soybeans (a) and black soybeans (b).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

HPLC Analysis of Isoflavones

The isoflavones of soybeans were extracted and analyzed according to the method of Lin and Lai (*13*) with some modifications. A Hitachi HPLC equipment (Hitachi Ltd., Japan) and a YMC-pack ODS-AM-303 column (250 mm × 4.6 mm i.d., 5 µm, YMC Co., Ltd., Japan) were used for the determinations. A linear gradient system of 0.01% TFA in acetonitrile (A) and 0.01% TFA in distilled water (B) is programmed as follows: from 0 to 20 min, eluent A is increased from 15 to 20%; from 20 to 30 min, eluent A was from 20 to 24%; from 30 to 34 min, eluent A was kept in 24%; from 34 to 44 min, eluent A was from 24 to 35%; from 44 to 60 min, eluent A was from 35 to 40%. The isoflavone isomers were identified by comparing the retention times and quantified by using standard curves with authentic standards; daidzein, daidzin, acetyldaidzin, genistein, genistin, acetylgenistin, malonylgenistin, malonylglycitin (LC Laboratories Co., Woburn, MA), glycitein, glycitin, acetylglycitin, and malonyldaidzin (Nagara Science Co., Ltd., Gifu, Japan).

Statistical Analysis

All assays were run at least in duplicate and the results were analyzed by analysis of variance (ANOVA) using the general linear model and Duncan's new multiple-range test ($p<0.05$), utilizing SAS software (SAS Institute, Inc., USA).

Results and Discussion

Total Isoflavones of Soybean after Soaking and Germination

Total isoflavone contents and amounts of isoflavone isomers of matured soybeans were significantly different among soybean cultivars, ranging from 5393 µg/g dry meal (KS1) to 1991 µg/g dry meal (KS8). The soaking and germination processes did increase the total amounts of isoflavones (Table 1 and Table 2) and the results agreed with previous reports (*13*, *18*). Total isoflavones increased (9-85%) after soaking and germination compared to their matured soybeans, e.g. malonyl glucosides were significantly increased by 54% for germinated soybean KS8 and aglycones were increased from 0 to 92 µg/g dry meal for germinated black soybean TN3. In general, the glucosides and acetyl glucosides slightly increased after soaking but decreased after germination. The total isoflavones of soaked and germinated soybeans ranged from 2902 to 5879 and 2650 to 6207 µg/g dry meal, respectively. During soaking and germination the biosynthesis of isoflavones was stimulated and the rate of biosynthesis would be affected by enzymatic activity of soybeans, which depending on the natural factors of dry soybeans. Previous reports indicated that the addition of β-glucosidase inhibitor would suppress the conversions of aglycones in soaked soybeans (*19*, *20*).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Table 1. Moist-thermal effect on the total contents of isoflavone subgroups in matured, soaked and germinated soybean KS1 and KS8

Variety	*Type*	*Time (min)*	*Aglycosides*	*Glucosides*	*Acetylglucosides*	*Malonylglucosides*	*Total*
			µg/g dry meal				
KS1	Matured	0	6^{a1}	1937^{a}	201^{a}	3250^{d}	5393
		10	26^{b}	2879^{b}	1125^{b}	589^{c}	4619
		20	32^{c}	3270^{c}	1210^{c}	155^{b}	4667
		30	41^{d}	3522^{d}	1322^{d}	27^{a}	4912
		60	62^{e}	3708^{e}	1385^{e}	--	5156
	Soaked	0	156^{a}	1624^{a}	396c	3703^{e}	5879
		10	182^{b}	3085^{b}	265^{a}	937^{d}	4470
		20	187^{b}	3717^{c}	315^{b}	406^{c}	4623
		30	230^{d}	3933^{d}	388^{c}	152^{b}	4703
		60	198^{c}	3776^{c}	317^{b}	3^{a}	4294
	Germinated	0	224^{d}	1388^{a}	76^{a}	4519^{e}	6207
		10	239^{d}	3577^{d}	295^{b}	899^{d}	5009
		20	185^{c}	3199^{b}	316^{b}	356^{c}	4056
		30	152^{a}	3461^{c}	371^{c}	137^{b}	4108
		60	175^{b}	4094^{e}	334^{bc}	8^{a}	4611
KS8	Matured	0	--^{2}	440^{a}	75^{a}	1475^{c}	1991
		10	7^{a}	1141^{b}	484^{b}	148^{b}	1780
		20	9^{a}	1306^{d}	543^{c}	35^{a}	1894
		30	9^{a}	1299^{cd}	551^{c}	--	1858
		60	16^{b}	1267^{c}	462^{b}	--	1740
	Soaked	0	--	1483^{a}	188^{a}	1567^{d}	3238
		10	--	2732^{c}	267^{b}	355^{c}	3355
		20	--	2601^{b}	285^{bc}	183^{b}	3069
		30	--	2782^{d}	300^{c}	123^{a}	3205
		60	--	2778^{d}	261^{b}	--	3038
	Germinated	0	--	341^{a}	129c	2279^{e}	2750
		10	--	1334^{b}	37^{a}	291^{d}	1662
		20	--	1770^{c}	70^{b}	135^{b}	1946
		30	--	3080^{d}	379^{e}	193^{c}	3652

Continued on next page.

Table 1. (Continued). Moist-thermal effect on the total contents of isoflavone subgroups in matured, soaked and germinated soybean KS1 and KS8

Variety	*Type*	*Time (min)*	*Aglycosides*	*Glucosides*	*Acetylglucosides*	*Malonylglucosides*	*Total*
			µg/g dry meal				
		60	--	3327^{e}	322^{d}	42^{a}	3691

[1] Mean values with the different superscript letters in each column within the thermal treatment were significantly different (p<0.05). [2] The amount is trace and expressed as zero for calculating the total isoflavones.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Moist-Thermal Effects on the Conversion of Isoflavone Subgroups

Depending on the soybean variety and the duration of moist-thermal treatment, the isoflavone isomers showed different profiles before and after moist thermal treatments. In general, the significant losses of malonyl glucosides, corresponding with the increases of glucosides, were occurred in the early heating stage (in 10 min) and resulted in the losses of 75-96% of malonyl glucosides and the increases of 49-333% of glycosides (Table 1 and Table 2). Chiarello et al. (*21*) reported that the total isoflavone contents of soymilk decreased (ca. 20%) after autoclaving at 121 °C for 15 min. The conversions of isoflavone subgroups during early stage of moist-thermal process were attributed to the thermal instability of malonyl glucosides, which might thermally degraded into respondent glucosides and acetyl glucosides due to the breakages of ester bonds and decarboxylation upon the heating (*22*). Chiarello et al. (*21*) also indicated that a 90% decrease in malonyl forms, whereas β-glycosides increased by 70% when soymilk was autoclaved for 5 min at 121 °C.

The moist-thermal effects on the changes of aglycone contents varied dramatically among the bio-status of starting materials, such as matured, soaked, or germinated soybean seeds. Aglycones were induced by heating when matured soybeans were thermal-treated. In contrast to matured soybeans, aglycones which induced during germination and soaking seemed thermal-labile after long duration of moist thermal-treatment (60 min), which led 22% loss in germinated soybean KS1 (Table 1) and 49% loss in germinated black soybean TN3 (Table 2). The similar phenomenon was reported by Huang and Chou (*23*), who indicated that steaming at 60 °C or higher for 30 min led to the loss of aglycones in black soybeans powder. Huang et al. (*16*) studied that prolonging heating time caused the thermal degradation of aglycones. It was suggested that the declines of aglycones in heat processed soybean products was attributed to Maillard reaction with soy protein (*16*, *24*). No aglycones could be determined in soaked and germinated soybean KS8 were attributed to low endogenous enzymatic activity of seeds after a long period of storage (2 years).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Table 2. Moist-thermal effect on the total contents of isoflavone subgroups in matured, soaked and germinated soybean TN3 and TN6

Variety	*Type*	*Time (min)*	*Aglycosides*	*Glucosides*	*Acetylglucosides*	*Malonylglucosides*	*Total*
			µg/g dry meal				
TN3	Matured	0	--[1]	624^{a}	110^{a}	1919^{c}	2654
		10	3^{a}[2]	1465^{b}	578^{b}	195^{b}	2240
		20	7^{a}	1870^{c}	694^{c}	46^{a}	2613
		30	7^{a}	2067^{d}	762^{d}	20^{a}	2857
		60	9^{a}	1891^{c}	682^{c}	22^{a}	2604
	Soaked	0	11	397^{a}	125^{d}	2368^{d}	2902
		10	--	1286^{b}	45^{a}	266^{c}	1557
		20	--	1907^{c}	77^{bc}	121^{b}	2186
		30	--	1931^{c}	97^{cd}	36^{a}	1863
		60	--	2243^{d}	273^{e}	--	2524
	Germinated	0	92^{b}	528^{a}	48^{a}	1981^{c}	2650
		10	109^{bc}	1899^{b}	134^{b}	89^{b}	2230
		20	110^{bc}	1910^{b}	137^{b}	18^{a}	1494
		30	107^{bc}	2014^{c}	339^{d}	--	2959
		60	47^{a}	1944^{b}	245^{c}	--	2181
TN6	Matured	0	--	795^{a}	100^{c}	1881^{c}	2776
		10	3^{a}	1929^{b}	66^{b}	221^{b}	2994
		20	8^{a}	2229^{d}	85^{bc}	19^{a}	3253
		30	11^{a}	2286^{d}	59^{ab}	--	3228
		60	18^{a}	2135^{c}	28^{a}	--	2976
	Soaked	0	8^{a}	702^{a}	121^{a}	2533^{d}	3364
		10	--	1846^{b}	191^{b}	647^{c}	2685
		20	--	2133^{d}	245^{c}	284^{b}	2661
		30	2^{a}	2281^{e}	296^{d}	63^{a}	2844
		60	4^{a}	1942^{c}	199^{bc}	--	1545
	Germinated	0	38^{a}	499^{a}	142^{a}	2596^{c}	3275
		10	55^{a}	2228^{c}	174^{a}	340^{b}	2798
		20	37^{a}	2139^{b}	217^{b}	52^{a}	2444
		30	55^{a}	2891^{e}	216^{b}	--	3072

Continued on next page.

Table 2. (Continued). Moist-thermal effect on the total contents of isoflavone subgroups in matured, soaked and germinated soybean TN3 and TN6

Variety	*Type*	*Time (min)*	*Aglycosides*	*Glucosides*	*Acetyl-glucosides*	*Malonyl-glucosides*	*Total*
			μg/g dry meal				
		60	76^{b}	2390^{d}	218^{b}	--	2684

[1] The amount is trace and expressed as zero for calculating the total isoflavones. [2] Mean values with the different superscript letters in each column within the thermal treatment were significantly different (p<0.05).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Dry-Thermal Effects on the Conversion of Isoflavone Subgroups

Similar conversion profiles of four isoflavone subgroups were investigated when soybeans were moist- and dry-thermal treated. The major difference was the higher conversion ratio in dry-thermal treated soybeans (Table 3 and 4) than that in moist-thermal treated soybeans (Table 1 and 2). In additions, the short dry-heating time (min) could effectively increase the amount of aglycones in soybeans, which increased 23 times (KS1) to 51 times (KS8) of their matured seeds (Table 1). The amounts of aglycones, in general, increased with extending the duration of thermal treatments. The malonyl glucosides dramatically decreased in matured soybeans or totally disappear in matured black soybeans when matured soybeans or black soybeans were 10 min dry-thermal treated. At the same time, the amounts of acetyl-glucosides dramatically increased, being attributed to the decarboxylation of malonyl glucosides. Mahungu et al. (*25*) mentioned the malonyl glucosides were the most susceptible and converted to the acetyl derivatives through heat-induced decarboxylation during extrusion. Coward et al. (*1*) also reported that malonyl groups were decarboxylated and large amounts of acetyl glucosides were formed in toasted soy flour. When the dry heating time increased to 20 min, the amount of acetyl glucosides of matured soybeans decreased. This might be resulted from the higher activation energy required for the breakage of ester bond than that for the decarboxylation. As the results, a 30 min dry-thermal treatment significantly increased the aglycones of matured soybeans although the total isoflavones contents slightly reduced. Aglycones contained principally daidzein and genistein in dry-thermal treated matured seeds, which ranged from 376 (KS 8) to 754 (TN 6) μg/g dry meal and 189 (KS 8) to 458 (TN 6) μg/g dry meal after dry-thermal treated for 30 min, respectively. The total isoflavones of dry-thermal treated soybeans reflected the combined effects of conversions of isoflavone subgroups upon heating.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Table 3. Dry-thermal effect on the total contents of isoflavone subgroups in matured, soaked and germinated soybean KS1 and KS8

Variety	*Type*	*Time (min)*	*Aglycosides*	*Glucosides*	*Acetylglucosides*	*Malonylglucosides*	*Total*
			µg/g dry meal				
KS1	Matured	0	6^{a}[1]	1937^{a}	201^{a}	3250^{b}	5393
		10	25^{a}	1943^{a}	1225^{b}	1582^{a}	4774
		20	185^{b}	2473^{c}	1956^{d}	--	4614
		30	603^{c}	2306^{b}	1668^{c}	--	4578
	Soaked	0	156^{b}	1624^{c}	396^{c}	3703^{d}	5879
		10	73^{a}	906^{a}	66^{a}	3020^{c}	4065
		20	69^{a}	1208^{b}	163^{b}	2656^{b}	4094
		30	649^{c}	1923^{d}	1412^{d}	483^{a}	4468
	Germinated	0	224^{b}	1388^{d}	76^{a}	4519^{d}	6207
		10	69^{a}	983^{a}	196^{b}	3833^{c}	5082
		20	111^{a}	1201^{c}	169^{b}	3234^{b}	4715
		30	85^{a}	1088^{b}	59^{a}	1482^{a}	2714
KS8	Matured	0	--[2]	435^{b}	75^{a}	1475^{b}	1985
		10	11^{a}	945^{d}	1015^{d}	197^{a}	2168
		20	234^{b}	829^{c}	771^{c}	--	1834
		30	573^{c}	38^{a}	439^{b}	--	1391
	Soaked	0	--	1483^{d}	188^{b}	1567^{c}	3238
		10	4^{a}	226^{a}	100^{a}	1570^{c}	1900
		20	1^{a}	396^{b}	131^{a}	637^{b}	1165
		30	10^{a}	532^{c}	258^{c}	412^{a}	1212
	Germinated	0	--	341^{a}	129^{a}	2279^{d}	2749
		10	17^{a}	404^{b}	200^{b}	1806^{c}	2427
		20	27^{a}	679^{c}	279^{c}	1349^{b}	2337
		30	94^{b}	900^{d}	814^{d}	176^{a}	1984

[1] Mean values with the different superscript letters in each column within the thermal treatment were significantly different ($p<0.05$). [2] The amount is trace and expressed as zero for calculating the total isoflavones.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Table 4. Dry-thermal effect on the total contents of isoflavone subgroups in matured, soaked and germinated soybean TN3 and TN6

Variety	*Type*	*Time (min)*	*Aglycosides*	*Glucosides*	*Acetyl-glucosides*	*Malonyl-glucosides*	*Total*
		μg/g dry meal					
TN3	Matured	0	--[1]	624^{b}	110^{a}	1919^{a}	2654
		10	18^{a}[2]	1124^{c}	1301^{c}	--	2443
		20	552^{b}	693^{b}	982^{b}	--	2227
		30	875^{c}	381^{a}	945^{b}	--	2201
	Soaked	0	11^{a}	397^{a}	125^{b}	2368^{d}	2902
		10	--	521^{b}	63^{a}	2120^{c}	2705
		20	--	1113^{d}	154^{b}	1067^{b}	2334
		30	21^{a}	995^{c}	762^{c}	53^{a}	1831
	Germinated	0	92^{a}	528^{a}	48^{a}	1981^{c}	2650
		10	162^{b}	788^{b}	136^{b}	1791^{b}	2876
		20	92^{a}	906^{c}	217^{c}	2056^{d}	3270
		30	121^{ab}	808^{b}	408^{d}	572^{a}	1909
TN6	Matured	0	--	795^{b}	100^{a}	1881^{a}	2776
		10	28^{a}	1033^{c}	1048^{d}	--	2108
		20	863^{b}	853^{b}	986^{c}	--	2701
		30	1330^{c}	223^{a}	393^{b}	--	1946
	Soaked	0	8^{a}	702^{b}	121^{a}	2533^{d}	3364
		10	--	638^{a}	--	1339^{c}	1977
		20	--	701^{b}	131^{a}	1052^{b}	1885
		30	60^{b}	1424^{c}	987^{b}	463^{a}	2933
	Germinated	0	38^{a}	499^{a}	142^{c}	2596^{c}	3275
		10	53^{a}	582^{b}	68^{a}	2989^{d}	3692
		20	70^{ab}	574^{b}	19^{a}	1763^{b}	2426
		30	89^{b}	1089^{c}	673^{d}	1045^{a}	2896

[1] The amount is trace and expressed as zero for calculating the total isoflavones. [2] Mean values with the different superscript letters in each column within the thermal treatment were significantly different ($p<0.05$).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

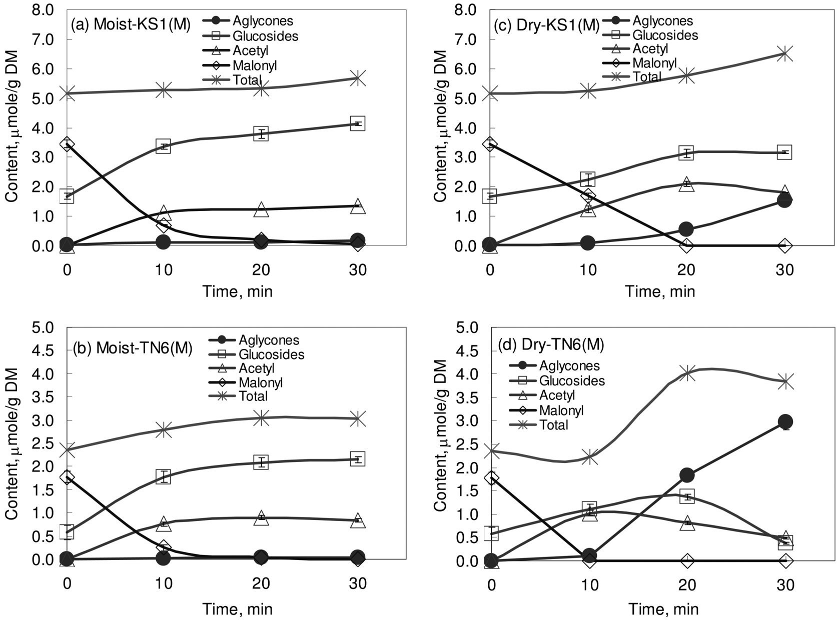


Figure 2. The interconversions of daidzein conjugates in matured soybean (KS1(M)) and matured black soybean (TN6(M)) during moist- (a & b) and dry-thermal treatments (c & d).

Thermal Effects on the Interconversions of Isoflavone Conjugates

The thermal stability and interconversion of daidzein conjugates in matured soybeans were shown in Figure 2. When soybeans were moist-heated at 121 °C, the total amounts of daidzein conjugates did not change significantly (Figure 2 (a) and (b)). The malonyldaidzin almost disappeared while the level of acetyldaidzin increased and daidzin was generated after 10 min heating. The stability and level of daidzein in soybeans was not affected by moist-heating at 121 °C. This result further demonstrated that malonyldaidzin could be converted to acetyldaidzin or daidzin during moist-thermal treatment at 121 °C.

When soybeans were dry-heated at 195 °C for 30 min, the total amounts of daidzein conjugates increased with increasing in heating time and the degree of increase was dependent on the soybean variety (Figure 2 (c) and (d)). The malonyldaidzin could not be detected in matured soybean KS1 and black soybean TN6 after dry-heating for 20 and 10 min, respectively. A similar outcome was observed for the conversions of acetyldaidzin and daidzin during dry-thermal treatment, which the levels of acetyldaidzin and daidzin increased after 10 min heating. In contrast to the moist-heating, the levels of acetyldaidzin and daidzin decreased after 20 min heating. Meanwhile, daidzein started to be generated after 10 min heating and followed by a significant increase, 1.53 and 2.97 μmole/g (db) in soybean KS1 and black soybean TN6 after heating for 30 min. This result indicated that acetyldaidzin and daidzin could be effectively converted to

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

daidzein when dry-heating was treated long enough. Eisen et al. (*26*) presented the results during soymilk processing that acetyldaidzin increased at early stages, followed by a slow decrease.

A similar outcome was observed for the stability and interconversion of genistein conjugates in matured soybeans during moist- and dry-thermal treatments (Figure 3). As described above, malonylgenistin is thermal labile, which is easily converted to acetyl- and glucosyl-genistin when both moist- and dry-heating is applied. During dry-heating at 195 °C up to 10 min, a loss of malonylgenistin and a gain of acetylgenistin were observed as described in soybeans treated with moist-heating at 121 °C for 30 min. After dry-heating at 195 °C for 20 min, acetylgenistin and genistin were further degraded into genistein, 0.79 and 1.69 μmole/g (db) in soybean KS1 and black soybean TN6.

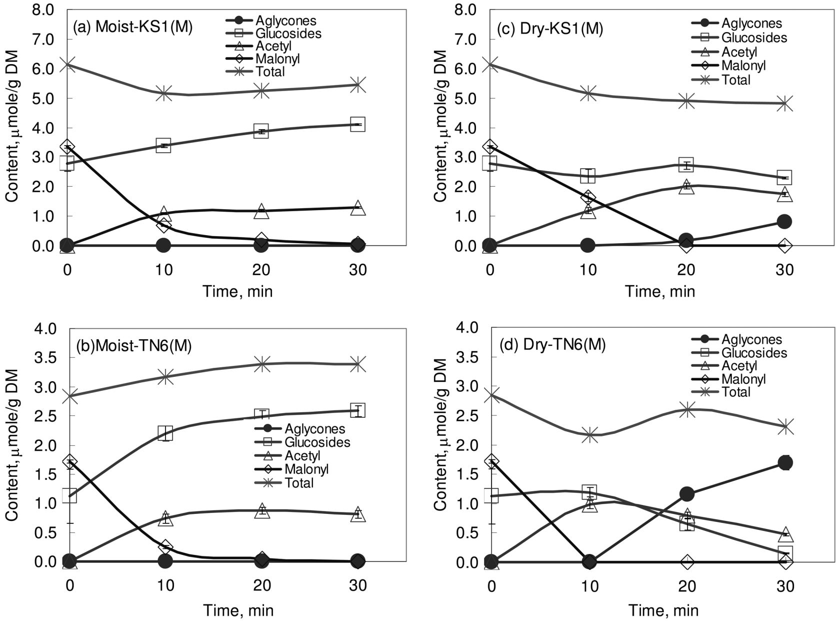


Figure 3. The interconversions of genistein conjugates in matured soybean (KS1(M)) and matured black soybean (TN6(M)) during moist- (a & b) and dry-thermal treatments (c & d).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

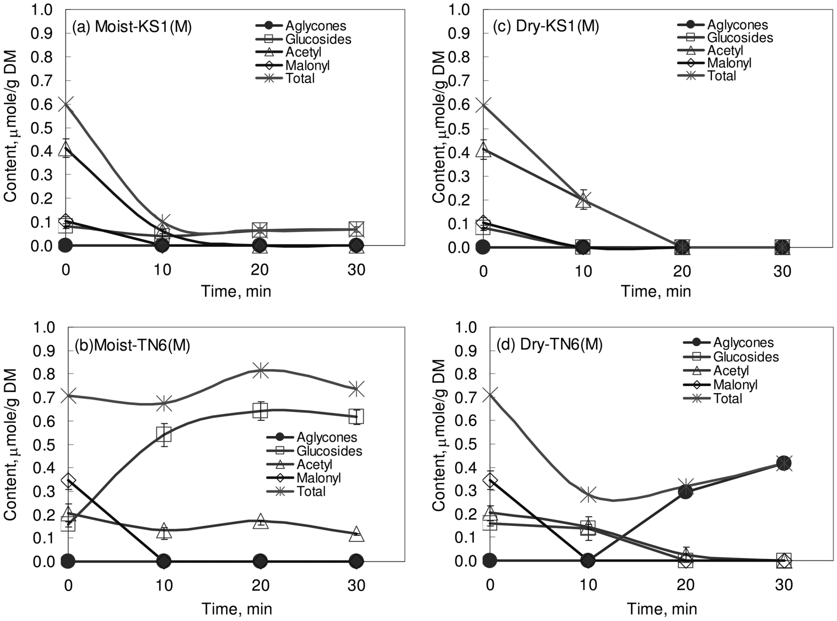


Figure 4. The interconversions of glycitein conjugates in matured soybean (KS1(M)) and matured black soybean (TN6(M)) during moist- (a & b) and dry-thermal treatments (c & d).

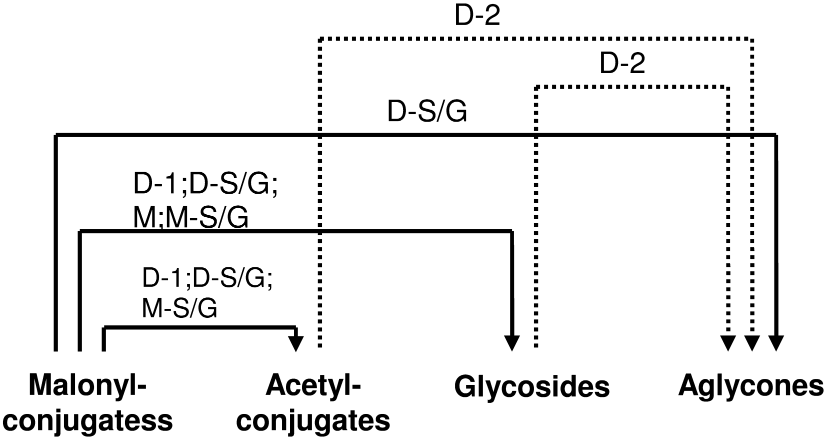


Figure 5. The outline diagram of interconversion pathways of isoflavone conjugates during thermal treatments. M: conversion during moist-thermal treatment of matured seeds; D-1: conversion during the early dry-thermal stage of matured seeds; D-2: conversion during the late dry-thermal stage of matured seeds; M-S/G: conversion during moist-thermal treatment of soaked & germinated seeds; D-S/G: conversion during dry-thermal treatment of soaked & germinated seeds.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Compared to the total amounts of daidzein conjugates (5.15 and 2.35 μmole/g (db) in soybean KS1 and black soybean TN6) and genistein conjugates (6.12 and 2.84 μmole/g (db) in soybean KS1 and black soybean TN6), the total amount of glycitein conjugates (0.60 and 0.71 μmole/g (db) in soybean KS1 and black soybean TN6) in matured soybeans was very low (Figure 4). The thermal stability of glycitein conjugates was, in general, low in matured soybean KS1 during moist- and dry-thermal treatments. Similar to malonyldaidzin and malonylgenistin, a loss of malonylglycitin during early heating stage was observed, while the corresponding increases of acetylglycitin and glycitin were not observed in both moist- and dry-thermal treated matured soybean KS1. After moist-heating for 10 min, an increase amount of glycitin in black soybean TN6 (Figure 4 (b)) was probably mainly from the conversion of malonylglycitin. After dry-heating for 30 min, only glycitein could be detected in black soybean TN6 with half amount of total glycitein conjugates in matured seeds. In the literatures, daidzein was more labile to degradation than genistein and glycitein in both standard solutions and thermal treated tofu (*16*, *27*). However, it was not consistent with the results in soybean seeds as shown in this study; glycitein conjugates were more sensitive to thermal treatment.

According to the results, the outline of interconversions of isoflavone conjugates was shown in Figure 5. During moist-heating, malonyl glycosides were mainly degraded into glycosides in matured seeds, and into glycosides and acetyl glycosides in soaked and germinated seeds. During dry-thermal treatment, malonyl glycosides in soaked and germinated seeds could convert into aglycones, glycosides, and acetyl conjugates. The degradation of malonyl glycoside in dry-heated matured seed was divided into two stages. Glycosides and acetyl glycosides were generated in early heating stage; aglycones were formed from the degradation of acetyl- and glycosides in late heating stage.

Conclusion

Many factors, resulting from the bio-status of soybeans after storage and pretreatments (soaking or/and germination), affected the profiles of isoflavones conjugates interconversion during thermal processing (moist- or dry-thermal treatment for various heating times). In most of soybeans, the soaking and germination can increase the amount of total isoflavones and aglycones. The moist-thermal (121 °C/60 min) and dry-thermal (195 °C/30 min) processing are suitable for the matured soybeans to obtain high amounts of aglycone conjugates. However, the soaked and germinated soybeans are not suggested to be thermal-treated, especial for the long dry-thermal treatment, because the total isoflavones is significantly lost and aglycone conjugates is not significantly increased after thermal treatments.

Acknowledgments

This work was supported by the grants 94AS-12.1.3-FD-Z1 and 95AS-10.1.5-FD-Z1 from the Council of Agriculture Executive Yuan, Taiwan.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

References

1. Coward, L.; Smith, M.; Kirk, M.; Barnes, S. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S–1491S.
2. Albertazzi, P.; Purdie, D. W. The nature and utility of the phytoestrogens: A review of the evidence. *Maturita.* **2002**, *42*, 173–185.
3. Ewies, A. A. A. Phytoestrogens in the management of the menopause: Up-to-date. *Obstet. Gynecol. Surv.* **2002**, *57*, 306–313.
4. Ma, D. F.; Qin, L. Q.; Wang, P. Y.; Katoh, R. Soy isoflavone intake increases bone mineral density in the spine of menopausal women: Meta-analysis of randomized controlled trials. *Clin. Nutr.* **2008**, *27*, 57–64.
5. Messina, M.; Ho, S.; Alekel, D. L. Skeletal benefits of soy isoflavones: A review of the clinical trial and epiderniologic data. *Curr. Opin. Clin. Nutr. Metab. Care* **2004**, *7*, 649–658.
6. Weaver, C. M.; Cheong, J. M. K. Soy isoflavones and bone health: The relationship is still unclear. *J. Nutr.* **2005**, *135*, 1243–1247.
7. Arjmandi, B. H.; Smith, B. J. Soy isoflavones' osteoprotective role in postmenopausal women: Mechanism of action. *J. Nutr. Biochem.* **2002**, *13*, 130–137.
8. Kano, M.; Takayanagi, T.; Harada, K.; Sawada, S.; Ishikawa, F. Bioavailability of isoflavones after ingestion of soy beverages in healthy adults. *J. Nutri.* **2006**, *136*, 2291–2296.
9. Izumi, T.; Piskula, M. K.; Osawa, S.; Obata, A.; Tobe, K.; Saito, M.; Kataoka, S.; Kubota, Y.; Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J. Nutri.* **2000**, *130*, 1695–1699.
10. Xu, X.; Wang, H.-J.; Murphy, P. A.; Cook, L.; Hendrich, S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutri.* **1994**, *124*, 825–832.
11. Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994a**, *42*, 1666–1673.
12. Wang, H. J.; Murphy, P. A. Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem.* **1994b**, *42*, 1674–1677.
13. Lin, P. Y.; Lai, H. M. Bioactive compounds in legumes and their germinated products. *J. Agric. Food Chem.* **2006**, *54*, 3807–3814.
14. Vaidya, N. A.; Mathias, K.; Ismail, B.; Hayes, K. D.; Corvalan, C. M. Kinetic modeling of malonylgenistin and malonyldaidzin conversions under alkaline conditions and elevated temperatures. *J. Agric. Food Chem.* **2007**, *55*, 3408–3413.
15. Chien, J. T.; Hsieh, H. C.; Kao, T. H.; Chen, B. H. Kinetic model for studying the conversion and degradation of isoflavones during heating. *Food Chem.* **2005**, *91*, 425–434.
16. Huang, H.; Liang, H.; Kwok, K. C. Effect of thermal processing on genistein, daidzein and glycitein content in soymilk. *J. Sci. Food Agric.* **2006**, *86*, 1110–1114.

17. Barnes, S.; Coward, L.; Kirk, M.; Sfakianos, J. HPLC analysis of isoflavonoids and other phenolic agents from foods and from human fluids. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 263–273.
18. Zhu, D.; Hettiarachchy, N. S.; Horax, R.; Chen, P. Isoflavone contents in germinated soybean seeds. *Plant Foods Hum. Nutr.* **2005**, *60*, 147–151.
19. Matsuura, M.; Obata, A.; Fukushima, D. Objectionable flavor of soy milk developed during the soaking of soybeans and its control. *J. Food Sci.* **1989**, *54*, 602–605.
20. Toda, T.; Sakamoto, A.; Takayanagi, T.; Yokotsuka, K. Changes in isoflaovne compositions of soybean foods during cooking process. *Food Sci. Technol. Res.* **2000**, *6*, 314–319.
21. Chiarello, M. D.; Le guerroué, J. L.; Chagas, C. M. S.; Franco, O. L.; Biancini, E.; João, M. J. Influence of heat treatment and grain germination on the isoflavone profile of soy milk. *J. Food Biochem.* **2006**, *30*, 234–247.
22. Chang, S. K. C. Isoflavones from Soybeans and Soy Foods. In *Functional Foods: Biochemical and Processing Aspects*; Shi, J., Mazza, G., Le Maguer, M., Eds.; CRC Press: Boca Raton, FL, 2002; Vol. 2, pp 39−69.
23. Huang, R.-Y.; Chou, C.-C. Heating affects the content and distribution profile of isoflavones in steamed black soybeans and black soybean koji. *J. Agric. Food Chem.* **2008**, *56*, 8484–8489.
24. Davies, C. G. A.; Netto, F. M.; Glassenap, N.; Gallaher, C. M.; Labuza, T. P.; Gallaher, D. D. Indication of the Maillard reaction during storage of protein isolates. *J. Agric. Food Chem.* **1998**, *46*, 2485–2489.
25. Mahungu, S. M.; Diaz-Mercado, S.; Li, J.; Schwenk, M.; Singletary, K.; Faller, J. Stability of isoflavones during extrusion processing of corn/soy mixture. *J. Agric. Food Chem.* **1999**, *47*, 279–284.
26. Eisen, B.; Ungar, Y.; Shimoni, E. Stability of isoflavones in soy milk stored at elevated and ambient temperatures. *J. Agric. Food Chem.* **2003**, *51*, 2212–2215.
27. Ungar, Y.; Osundahunsi, O. F.; Shimoni, E. Thermal stability of genistein and daidzein and its effect on their antioxidant activity. *J. Agric. Food Chem.* **2003**, *51*, 4394–4399.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch011

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Chapter 12

Soy Protein Ingredients as Isoflavone Sources for Functional Foods

Maria Inés Genovese* **and Franco M. Lajolo**

Departamento de Alimentos e Nutrição Experimental,
Faculdade de Ciências Farmacêuticas,
Universidade de São Paulo, Av. Prof. Lineu Prestes 580,
Bloco 14, 05508-900, São Paulo, SP, Brasil
***Tel.: +5511-30913647. Fax: +5511-38154410. E-mail: Genovese@usp.br.**

The knowledge of the levels and profile of isoflavones present in soy protein ingredients, as well as the effect of industrial processing, is important for the development of functional foods rich in these compounds. Soy isoflavones, daidzein, genistein and glycitein, are present in seeds as glycosylated conjugates (malonylglycosides and underivatized β-glycosides); as a result of processing, aglycones and acetylglycosides can be formed. Total isoflavone content is in the range of 60 to 340 mg/100 g for soy ingredients such as defatted and whole soy flours (90-95% glycosylated), soy protein isolates (20-55% aglycones) and textured soy proteins (90-95% glycosylated, but 15-25% acetylglycosides). The highest isoflavone concentration is present in flours obtained from the hypocotyls, from 500 to 850 mg/100 g. Removal of lipids with hexane from the whole flour causes an increase in isoflavone concentration in defatted flours without altering the conjugation profile. However, extrusion process may provoke destruction of isoflavones and a significant increase in the amount of acetylglycosides. The production of soy isolates leads to an increase in the percentage of free aglycones. Soy beverages, whose consumption has been increasing in the last few years, present an isoflavone content varying from 10 to 80 mg/L. Frozen vegetarian dishes produced industrially with soy protein ingredients in substitution of meat have a concentration of 5 to 25 mg of isoflavones per 100 g (FW). However, the level of soy ingredients addition can

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

be increased, without affecting sensory attributes, in order to obtain isoflavone enriched-products.

Keywords: isoflavones; soy ingredients; functional foods

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Introduction

Isoflavones represent the most common group of phytoestrogens and have been associated to beneficial effects in humans, such as prevention of cancer, cardiovascular diseases, osteoporosis and menopausal symptoms (*1*). These bioactive substances are found in particularly high levels in soybeans, whose consumption is not part of Western eating habits as in Asian countries. The Japanese population, which consumes a high amount of soybeans and soybean-based processed foods, has a daily intake of isoflavones estimated as 28 mg/day (*2*), and for Asians in general it is of 20 to 80 mg/day. For Canadians and North-Americans, on the other hand, it is of~1-3 mg/d or below 1 mg/day in the absence of soy protein (*3*).

The interest in soy as a food has been increasing proportionally to the increase of evidences about its potential of reduction of several chronic diseases. Various epidemiological studies have demonstrated a lower incidence of breast, prostate and colon cancer in Asian populations, for which per capita soy consumption is 20 to 50 times higher than for occidental populations (*1*). On the other hand, soy protein products have been largely used as ingredients in meat products, breads, beverages, soups and other foods due to physicochemical properties such as water and oil retention. The FDA (Food and Drug Administration) approved in 1999 a health claim on food labels, for products containing soy protein, about the association between soy protein (25 g/d) and a reduced risk of coronary heart disease. In this way, there is an increasing interest of the food industry on the development of functional foods rich in soy protein and isoflavones.

Soy isoflavones, daidzein, genistein and glycitein, are present in the seed as glycosylated derivatives: the 7-O-β-(6″-O-malonyl)-D-glycosides (malonyldaidzin, malonylgenistin and malonylglycitin) and the de-esterified 7-O-β-glycosides (daidzin, genistin and glycitin). During soy processing, aglycones (daidzein, genistein and glycitein) and 7-O-β-(6″-O-acetyl)-D-glycosides (acetyldaidzin, acetylgenistin and acetylglycitin) can be formed as a result of enzymatic action and heating, respectively (*4*, *5*). The biological significance of the presence of these different forms in soybeans is not clear yet, but differences in their antioxidant activities and bioavailabilities were reported. (*6*–*8*).

After ingestion, isoflavones are absorbed after deglycosylation in the gastrointestinal tract by microbial β-glycosidases, and their estrogenic activity seems to be the result of the di-aryl nucleus similar to that of stilbene, hydroxylated at the 4′ and/or 7 positions (*9*). There is little information about the bioavailability of these different forms. It has been shown that the absorption of aglycones in humans was faster and more extensive than that of the glycosides (*10*). Contrarily to this, Setchell *et al.* (*7*) reported a higher bioavailability of glycosides compared to aglycones, and Xu *et al.* (*11*) observed no differences in bioavailability associated to isoflavone form.

Isoflavones in Soy Seeds

The isoflavone content of soybeans is highly variable, differing according to the variety, environmental and soil conditions (*12*). The analysis of 210 soybean cultivars from South Dakota (USA) showed isoflavone levels from 116 to 274 mg/100 g (*13*). Isoflavone contents from 118 to 175 mg/100 g were reported for the same crop of an American variety grown in three different locations (*14*). Differences between isoflavone profile of American and Japanese varieties have also been reported, the last with a higher content of malonylglycitin and also higher proportions between malonylgenistin and genistin and between malonyldaidzin and daidzin (*14*). The analysis of the isoflavone content of 15 different soy cultivars from the Paraná State (Brazil) showed an average concentration 31 % higher for the cultivars from the city of Ponta Grossa (120 mg/100 g) in comparison with those from Londrina (82 mg/100 g). These differences were attributed to differences in the temperature and soil composition between the two regions, and among the varieties analyzed the isoflavone content showed a higher variation, from 54 to 147 mg/100 g (*15*). Similarly, thirteen varieties and one new strain of soybeans, developed by the Genetic Improvement Program from EMBRAPA (Brazil), crop year of 2003, presented total isoflavone contents varying from 57 to 188 mg per 100 g of soybeans. Most (90-95 %) of the isoflavones were present as glycosylated derivatives. The β-glycosides represented the main forms of the isoflavones in the seed (50 to 59 % of the total) followed by malonylglycosides (28 to 39 % of the total). The ratio between malonyldaidzin and daidzin varied from 0.44 (Embrapa soyseeds BRS-155) to 0.74 (BRS-185) and between malonylgenistin and genistin from 0.56 (BRS-155) to 0.87 (BRS-185). The total percentage of each isoflavone, corresponding to the sum of the four chemical forms (malonylglycoside, acetylglycoside, β-glycoside and aglycone) varied from 39 to 57 %, for genistein, 34 to 47 %, for daidzein, and 8 to 17 % of the total, for glycitein (*16*).

The concentration of isoflavones is higher in hypocotyls (1400-1750 mg/100 g) than in cotyledons (160-320 mg/100 g) and hulls (10-20 mg/100 g), and the ratio among the three isoflavones also differs, of 4:5:1 in the cotyledons and of 4:1:3 (Total daidzein : Total genistein : Total glycitein) in the hypocotyls (*12*).

Isoflavones in Soy Protein Ingredients

The importance of soy products is that they represent a way of incorporating isoflavones in the diet of populations that are not used to the consumption of the grains, such as the Occidentals. Soy milk, as an example, can be used to elaborate flavored soymilk, yogurt, ice cream and tofu, and soy-nuts can be consumed as snacks and granola ingredients. Soy flours can be used in the preparation of bread high in phytoestrogens, whose daily consumption has been recently shown to

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

favorably influence PSA (prostate-specific antigen) levels in men diagnosed with prostate cancer after just one month (*17*).

Soy protein ingredients such as defatted soy flours, protein isolates, concentrates and textured proteins are already largely used in the food industry in meat products, breads, beverages, soups and other foods. The physicochemical properties of soy proteins such as geleification, water absorbing and emulsifying capacities help conferring texture and other important properties to the products (*18*). The composition of the main soy protein ingredients is presented in Table 1, and the processing for production is depicted in Figure 1.

Soybean flours are normally produced by a process consisting of cleaning, heating, cracking, dehulling, grinding and, for defatted flours, removing the oil with hexane. This is the starting material for most commercial soybean products such as protein isolates and concentrates (*18*).

The major isoflavones in unprocessed soybean- malonylgenistin, genistin, malonyldaidzin and daidzin- can be lost and/or transformed into other forms of isoflavones during processing (*22*). Important losses are observed during soaking, heating, filtration, and alkaline extraction, used in soy ingredients production (*5*, *23*, *24*). Aglycones are formed by the action of endogenous β-glycosidases during soy protein extraction or soy milk production. It has been shown that during soaking of soybeans maximum production of aglycones occurred at 45 ºC and pH 5.5, with almost complete inactivation of β-glycosidases at 60 ºC and pH below 4.3 or above 7 (*25*). Reaction rate was shown to be extremely rapid so that after 120 min ~93% of daidzin and genistin in soymilk were hydrolyzed (*25*). Storage was also found to alter isoflavone profile of soy protein ingredients (*26*).

The concentration and profile of isoflavones in soy based products and foods containing soy protein ingredients are determined by processing conditions (Figures 2 and 3) together with the content in soybean seeds, which as previously discussed is affected by genetic and environmental factors.

The isoflavone content of soy flours depends directly on that of the seeds used in their production. For whole flours it is in the range of 128 to 190 mg of isoflavones per 100 g FW, and for defatted flours, from 120 to 340 mg of isoflavones per 100 g FW (results expressed as aglycones).This increase is mostly due to concentration caused by oil removing, since hexane does not extract isoflavones due to the highly polar nature of the glycosidic conjugates and their inability to partition into the lipophilic oil during soy oil extraction (*4*). Umphress et al. (*27*) reported much lower isoflavone contents for defatted soy flours, ranging from 46 to 100 mg/100 g. This variation can be considered normal since different varieties present different contents of isoflavones and even the same variety can present differences depending on climate conditions and growth place (*12–15*). In both whole and defatted flours the main isoflavone forms present are malonylglycosides and deesterified β-glycosides, corresponding to 86 to 98 % of total isoflavones (*4*).

Commercial soy protein isolates are the most refined soy protein ingredient and are made from defatted flours/flakes by solubilizing protein at pH 6.8-10 and separating the extract, by centrifugation or filtration, from insoluble fibrous residues. The resulting supernatant is acidified (pH 4.5), to precipitate protein as a curd, and separated from soluble oligosaccharides by centrifugation. Then

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Table 1. Composition of soy protein products (%) and isoflavone contents (mg/100 g)*

	Moisture	*Protein*	*Lipids*	*Ash*	*Fiber*	*Isoflavones*
Full-fat flour	3.4	41.0	22.5	5.1	1.7	128-200
Defatted flour	6.5	53.0	1.0	6.0	3.0	120-381
Concen-trate	8.0	65.3	0.3	4.7	2.9	0**-135
Isolate	4.8	92.0	-	4.0	0.25	60-180

* Adapted from (*4*, *19–21*). ** Concentrates produced through alcohol washing of the defatted flour present no isoflavones, opposed to those produced through water (pH 4-5) wash.

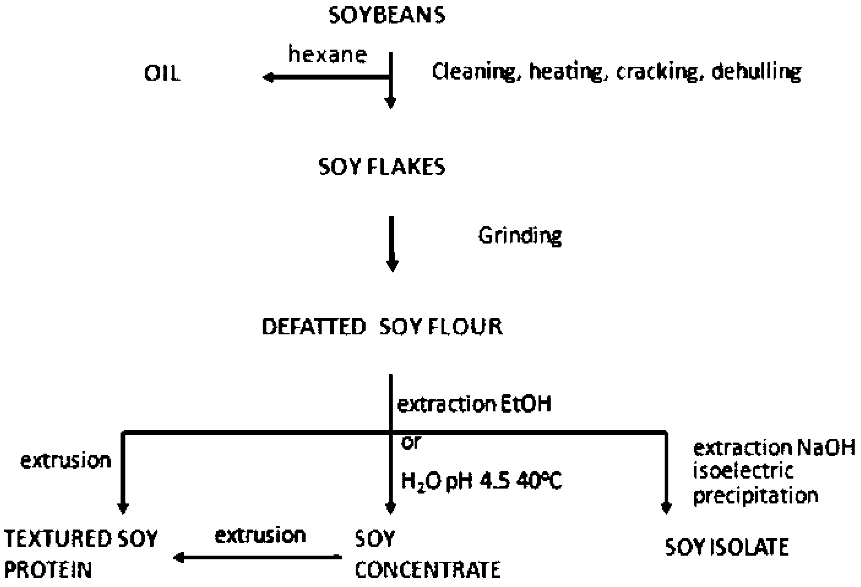

SOYBEANS
OIL
hexane
Cleaning, heating, cracking, dehulling
SOY FLAKES
Grinding
DEFATTED SOY FLOUR
extraction EtOH
or
H₂O pH 4.5 40°C
extrusion
extraction NaOH
isoelectric
precipitation
TEXTURED SOY PROTEIN
extrusion
SOY CONCENTRATE
SOY ISOLATE

Figure 1. Schematic representation of soy protein ingredients production.

the protein is directly spray-dried or it can be previously neutralized to pH 6.5-7 (*18*). The final product has a protein content of more than 90% and a variable isoflavone content, from 88 to 164 mg/100 g. The fiber-rich residue obtained after protein extraction from the defatted flour has also isoflavones (~ 50-60 mg/100 g), mainly in the form of aglycones (~70% of the total) (*4*).

Previously, it was shown that the defatted flour presented an isoflavone content (156 mg/100 g) 17.3% higher than that of the protein isolate produced from it (129 mg/100 g). The isoflavone conjugation was significantly altered, mainly in relation to the aglycone content, which increased from 4.5 to 31%, with a decrease of malonylglycosides and β-glycosides from 67 to 51% and 26 to 14% of the total, respectively. The increase in aglycones could be attributed to the action

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

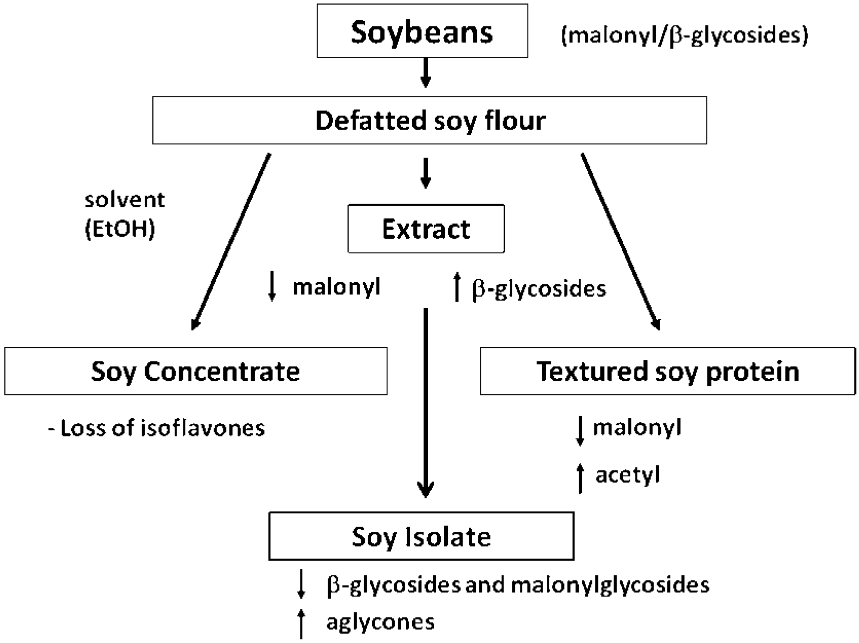

Soybeans
(malonyl/β-glycosides)
Defatted soy flour
solvent
(EtOH)
Extract
malonyl
β-glycosides
Soy Concentrate
- Loss of isoflavones
Textured soy protein
malonyl
acetyl
Soy Isolate
β-glycosides and malonylglycosides
aglycones

Figure 2. Schematic representation of the effect of soy protein ingredients production on isoflavones.

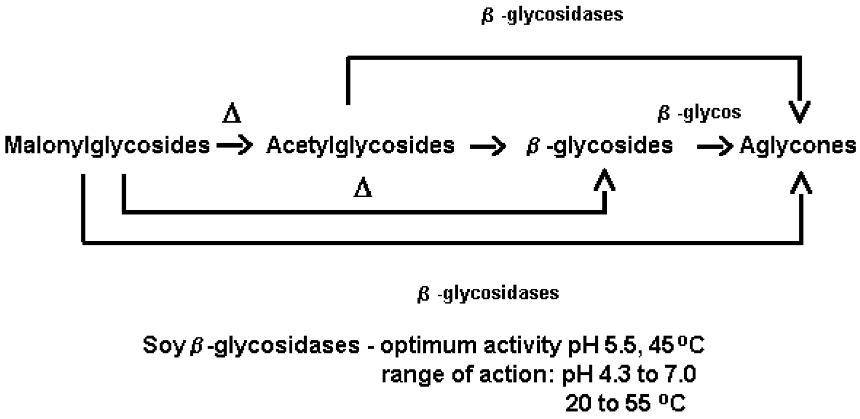

β -glycosidases
Δ
β -glycos
Malonylglycosides
Acetylglycosides
β -glycosides
Aglycones
Δ
β -glycosidases
Soy β -glycosidases - optimum activity pH 5.5, 45 °C
range of action: pH 4.3 to 7.0
20 to 55 °C

Figure 3. Effect of processing on soy isoflavones.

of endogenous β-glycosidases as it was previously shown that temperatures below 50 °C during aqueous protein extraction caused hydrolysis of β-glycosides with increase of aglycones, by soy β-glycosidase activity (*5*).

In the soy protein concentrate manufacture a water (pH 4-5) or alcohol wash of the defatted flour can be used to remove soluble carbohydrates and improve its functionality. The proteins that are not soluble in these conditions are concentrated in the residue (60-70% protein) after carbohydrates were removed. Alcohol washing results in the loss of most of the isoflavones, whereas a substantial amount of them are retained after acidified water washing, resulting in contents from 30 to 135 mg/100 g (*4*).

Soy hypocotyls, which represent 2% of the seed weight (*12*), became a very popular product for utilization in supplements of isoflavones due to their elevated isoflavone content, from 540 to 840 mg/100 g FW, mainly composed of malonylglycosides and deesterified β-glycosides, representing between 92 and 95% of the total forms. Differently from soy seeds and the other soy products, soy hypocotyls show a prevalence of glycitein and daidzein in relation to genistein and their respective conjugates (*4*).

Extrusion cooking is a heating process at high temperatures during a short time in which soy flour or protein concentrate change into texturized soy, characterized by the presence of isoflavones in the form of acetylglycosides (as a result of decarboxilation reactions), in amounts depending on the degree of exposition to heat during the processing, which can also cause a significant degradation of these compounds (*4*).

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Isoflavones in Soy Foods

Whole soy foods such as tofu, soy milk, miso and fresh soybeans are highly consumed among Asian population. Although the role of soybeans in the Brazilian diet is by far superseded by that of dry beans, the market of soy-based products has been increasing significantly in the last decade. Also, a high intake of soy-based products is observed for special groups such as vegetarians, lactose intolerants or allergic to milk proteins. Besides these groups, there are some products which are largely consumed by people in general, such as soy sauces and soy based beverages, these last becoming increasingly popular. The knowledge of the isoflavone content of these foods is important for those people interested in increasing isoflavone consumption from dietary sources.

Textured soy proteins are largely used by vegetarians as meat substitutes and present a high content of isoflavones, between 87 and 100 mg/100 g, slightly inferior to the values found previously by us for industrial samples (*28*). A mean value of 149 mg/100 g can be found in the USDA database on the isoflavone content of foods (*29*), with a significant range of variation, from 4 to 296 mg/100 g. In Brazil, the use of textured soy protein as a food ingredient in meat products is allowed until a maximum of 11.2% wet weight, and as a result the ingestion of 100 g of the final product would correspond to an ingestion of 9.7 - 11.2 mg of isoflavones. In this way, depending on the quantity and frequency of consumption of textured soy proteins, they can represent an important source of isoflavones in the diet. The same can be said about soy-nuts, prepared by frying previously blanched soy cotyledons in soybean oil until brown and crispy, which present the same isoflavone content of the starting material (*28*, *30*).

Soy beverages are becoming increasingly popular and are normally constituted of soymilk mixed with fruit juices and/or containing flavoring ingredients. Soymilk is obtained by water extraction of raw soybeans which were previously soaked in water, washed, and ground. The slurry is cooked and filtered to separate soymilk from the water insoluble residue (okara). Protein content can range from 0.6 to 2.5 g/100 mL, depending on the amount of fruit juice added, with the protein derived almost exclusively from soy. Total content of isoflavones in soy beverages varied significantly, ranging from 18 to 83 mg/L, or 2 to 4 mg of isoflavones per g of protein, and the most abundant compounds in all the beverages were the β-glycosides, representing 60 to 98% of the total (*28*). In this way, the consumption of just a glass of 250 mL of soy beverages could result in an intake of around 20 mg of isoflavones, or 0.29 mg/kg for a 70 kg-adult, almost equivalent to the Korean daily intake (21 mg/day) (*31*). Instant soy beverages consist basically of soymilk powder with flavoring ingredients, and present isoflavone contents from 40 to 50 mg/100 g. Much higher values, around 100 mg/100 g, were reported by Wang & Murphy (*32*) for four commercial soymilk powders. However, lower isoflavone contents, of approximately 9 and 17 mg/100 g, were found by Barnes *et al.* (*33*) in two soymilks analyzed. A mean value of 110 mg/100 g (ranging from 100 to 125) is reported in the USDA database on the isoflavone content of foods (*29*). These differences are probably the result of differences in the isoflavone content of soybeans used for soymilk production and the amount of other ingredients added to instant soy beverages.

Although being traditional Asian soy foods, shoyu, tofu and miso are frequently consumed by Western populations also. Shoyu is a soy sauce produced through fermentation of a paste of soybeans, rice and other cereals and has a mean content of 1.6 mg of isoflavones/100 g (*29*). Tofu is produced by precipitation of a curd from soymilk with calcium salts and removal of fluid by pressing. An isoflavone content ranging from 20 to 35 mg/100g fw was previously reported for 12 different samples of tofu, from regular to extra firm kinds (*34*), but the extra soft kind presented lower isoflavone content, of 7 mg/100 g fw (*28*). Miso (made of fermented rice and soybeans) presents a significant amount of isoflavones, of 23 to 89 mg/100 g, with a high amount of aglycones, formed by the action of exogenous β-glycosidases during fermentation (*28*, *29*, *32*, *35*). Taking in consideration that soy sauce and miso are consumed in very low amounts usually as seasonings, their contribution to the intake of isoflavones would be insignificant. Although the daily intake of tofu can be quite high - 40 g per day for Japanese people (*2*) – the low content of isoflavone results in a poor contribution for the total intake of these compounds.

Soy based infant formulas are lactose-free preparations indicated in case of galactosemia, intolerance to lactose, and allergy to cow milk. Their protein content is of about 15% and corresponds exclusively to isolated soy proteins. For soy based infant formulas commercialized in Brazil it was found a total isoflavone content ranging from 7.4 to 24 mg per 100 g of powder, expressed as aglycones. The total isoflavone content found for six soy based infant formulas commercialized in the U.S.A. showed a lower variation and higher values, from 21 to 29 mg per 100 g of powder (*36*). The daily intake of isoflavones of infants consuming soy formulas was estimated between 5.9 and 35 mg per day according to the product and age of the infant. Considering the average body weights, for infants 0 to 2 week-old, the isoflavone daily intake would be of 2.0 to 6.1 mg/kg, for those 2-8 week-old, 1.7 to 6.6 mg/kg, for those 2-3 month-old, 1.6 to 5.2 mg/kg, and for those 3-6 month-old, of 1.4 to 5.4 mg/kg. These doses correspond to a daily intake between 98 and 462 mg of isoflavones for a 70 kg-adult (*28*).

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

New soy-containing products found in the Brazilian market present a large variation of isoflavone contents, from ~5 mg/100 g FW, for soy bread, vegetarian *lasagna* and vegetarian hamburger, to ~25 mg/100 g FW for vegetarian *kibe* (unpublished results).

Among the products mentioned here, the isoflavone content of soy based infant formulas can be considered high taking in account their elevated daily intake as mg/kg of body weight. In the absence of clinical studies demonstrating that the potential beneficial effects observed for adults could be extended to children, it would be advisable to reconsider their utilization in presence of hormonal disruption. For those individuals who are willing to increase isoflavone intake, the consumption of soy seeds or other soy based foods is recommended. Textured soy proteins and some of the soy based beverages present high isoflavones contents, representing potential sources of isoflavones in our diet. In almost all the products analyzed the β-glycosides were the predominant form of the isoflavones, exception made to the products of fermentation miso and shoyu in which the aglycones were present in the highest proportions. Although soy beverages and textured soy proteins may represent important sources of isoflavones in our diet, the amount of isoflavones that humans would need to consume to provide an anticarcinogenic dose was estimated as 1.5-2.0 mg/kg of body weight per day (*37*), which means that having soy beverages as the sole isoflavone source the consumption of at least 5 glasses of would be necessary. On the other hand, a daily intake of around 40-50 mg seems to be enough to have benefits in preventing/treating osteoporosis (*38*).

Concluding Remarks

Nowadays, the main concern related to the use of soy protein ingredients as isoflavone sources is that different protein derivatives and different lots of the same product can present a great variation in the content and profile of isoflavones, related to variations in the raw material and processing conditions. For the development of functional foods, soy hypocotyls were shown to represent the richest sources of isoflavones, followed by defatted flours. Soy fibers are good sources of isoflavones in the aglycone form, and associated to the beneficial physiological effects of dietary fibers, they could be also interesting for inclusion in functional foods. Products derived from hypocotyls were shown to be richer in daidzein and glycitein conjugates. Malonylglycosides seem to be the most sensitive forms to processing, being subjected to both decarboxilation and deesterification reactions, forming the respective acetylglycosides and β-glycosides. The biological significance of the kind and conjugation of isoflavones, which differs deeply among soy products, remains to be determined.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

References

1. Adlercreutz, H.; Mazur, W. Phyto-oestrogens and Western diseases. *Ann. Med.* **1997**, *29*, 95–120.
2. Nakamura, Y.; Tsuji, S.; Tonogai, Y. Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake. *J. AOAC Int.* **2000**, *83*, 635–650.
3. Wu, A. H.; Yu, M. C.; Tseng, C. C.; Pike, M. C. Epidemiology of soy exposures and breast cancer risk. *Brit. J. Cancer* **2008**, *98*, 9–14.
4. Genovese, M. I.; Barbosa, A. C. L.; Pinto, M. S.; Lajolo, F. M. Commercial soy protein ingredients as isoflavone sources for functional foods. *Plant Foods Hum. Nutr.* **2007**, *62*, 53–58.
5. Barbosa, A. C. L.; Lajolo, F. M.; Genovese, M. I. Influence of temperature, pH and ionic strength on the production of isoflavone-rich soy protein isolates. *Food Chem.* **2006**, *98*, 757–766.
6. Naim, M.; Gestetner, B.; Bondi, A.; Birk, Y. Antioxidative and antihemolytic activities of soybean isoflavones. *J. Agric. Food Chem.* **1976**, *24*, 1174–1177.
7. Setchell, K. D. R.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirschner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, *131*, 1362S–1375S.
8. Xu, X.; Wang, H. J.; Murphy, P. A.; Cook, L.; Hendrich, S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutr.* **1994**, *124*, 825–832.
9. Dodge, J. A. Natural and anthropogenic environmental oestrogens: The scientific basis for risk assessment. *Pure Appl. Chem.* **1998**, *70*, 1725–1733.
10. Izumi, T.; Piskula, M. K.; Osawa, S.; Obata, A.; Tobe, K.; Saito, M.; Kataoka, S.; Kubota, Y.; Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J. Nutr.* **2000**, *130*, 1695–1699.
11. Xu, X.; Wang, H. J.; Murphy, P. A.; Hendrich, S. Neither background diet nor type of soy food affects short-term isoflavone bioavailability in women. *J. Nutr.* **2000**, *130*, 798–801.
12. Eldridge, A. C.; Kwolek, W. F. Soybean isoflavones: Effects of environment and variety on composition. *J. Agric. Food Chem.* **1983**, *31*, 394–396.
13. Wang, C.; Sherrard, M.; Pagadala, S.; Wixon, R.; Scott, R. A. Isoflavone content among maturity group 0 to II soybeans. *J. Am. Oil Chem. Soc.* **2000**, *7*, 483–487.
14. Wang, H.; Murphy, P. A. Isoflavone composition of American and Japanese soybean in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem.* **1994**, *42*, 1674–1677.
15. Carrão-Panizzi, M. C.; Keisuke, K.; Beléia, A. D. P.; Oliveira, M. C. N. Influence of growth locations on isoflavone contents in Brazilian soybean cultivars. *Breed. Sci.* **1998**, *48*, 409–413.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

16. Genovese, M. I.; Hassimotto, N. M. A.; Lajolo, F. M. Isoflavone profile and antioxidant activity of Brazilian soybeans varieties. *Food Sci. Technol. Int.* **2005**, *11*, 205–211.
17. Dalais, F. S.; Meliala, A.; Wattanapenpaiboon, N.; Frydenberg, M.; Suter, D. A. I.; Thomson, W. K.; Wahlqvist, M. L. Effects of a diet rich in phytoestrogens on prostate-specific antigen and sex hormones in men diagnosed with prostate cancer. *Urology* **2004**, *64*, 510–515.
18. Garcia, M. C.; Marina, M. L.; Laborda, F.; Torre, M. Chemical characterization of commercial soybean products. *Food Chem.* **1998**, *62*, 325–331.
19. Wolf, W. J. Legumes: Seed Composition and Structure, Processing into Protein Products and Protein Materials. In *Food Proteins*; Whitaker, J. R., Tannenbaum, S. R. , Eds.; AVI: New York, 1977.
20. Genovese, M. I.; Lajolo, F. M. Determination of isoflavones in soy products. *Ciênc. Tecnol. Aliment.* **2001**, *21*, 86–93.
21. Barbosa, A. C. L.; Hassimotto, N. M. A.; Lajolo, F. M.; Genovese, M. I. Isoflavone content and profile and antioxidant activity of soy and soy products. *Ciênc. Tecnol. Aliment.* **2006**, *26*, 921–926.
22. Wang, H.; Murphy, P. A. Isoflavone content in commecial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673.
23. Jackson, C. J. C.; Dini, J. P.; Lavandier, C.; Rupasinghe, H. P. V.; Faulkner, H.; Poysa, V.; Buzzell, D.; Degrandis, S. Effects of processing on the content and composition of isoflavones during manufacturing of soy beverage and tofu. *Process Biochem.* **2002**, *37*, 1117–1123.
24. Rickert, D. A.; Meyer, M. A.; Murphy, P. A. Effect of extraction pH and temperature on isoflavone and saponin partitioning and profile during soy protein isolate production. *J. Food Sci.* **2004**, *69*, 623–631.
25. Matsuura, M.; Obata, A. β-Glucosidases from soybeans hydrolyze daidzin and genistin. *J. Food Sci.* **1993**, *58*, 144–147.
26. Pinto, M. S.; Lajolo, F. M.; Genovese, M. I. Effect of storage temperature and water activity on the content and profile of isoflavones, antioxidant activity, and in vitro protein digestibility of soy protein isolates and defatted soy flours. *J. Agric. Food Chem.* **2005**, *53*, 6340–6346.
27. Umphress, S. T.; Murphy, S. P.; Franke, A. A.; Custer, L. J.; Blitz, C. L. Isoflavone content of foods with soy additives. *J. Food Comp. Anal.* **2005**, *18*, 533–550.
28. Genovese, M. I.; Lajolo, F. M. Isoflavones in soy-based foods consumed in Brazil: Levels, distribution, and estimated intake. *J. Agric. Food Chem.* **2002**, *50*, 5987–5993.
29. USDA−Iowa State University Isoflavones Database. http://www.nal.usda.gov/fnic/foodcomp/ Data/isoflav.
30. Genovese, M. I.; Davila, J.; Lajolo, F. M. Isoflavones in processed soybean products from Ecuador. *Braz. Arch. Biol. Technol.* **2006**, *49*, 853–859.
31. Choi, Y.-S.; Lee, B.-H.; Kim, J.-H.; Kim, N.-S. Concentration of phytoestrogens in soybeans and soybean products in Korea. *J. Sci. Food Agric.* **2000**, *80*, 1709–1712.

32. Wang, H.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673.
33. Barnes, S.; Kirk, M.; Coward, L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC−mass spectrometry. *J. Agric. Food Chem.* **1994**, *42*, 2466–2474.
34. Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavones in retail and institucional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697–2704.
35. Franke, A. A.; Hankin, J. H.; Yu, M. C.; Maskarinec, G.; Low, S. H.; Custer, L. J. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. *J. Agric. Food Chem.* **1999**, *47*, 977–986.
36. Murphy, P. A.; Song, T.; Buseman, G.; Barua, K. Isoflavones in soy-based infant formulas. *J. Agric. Food Chem.* **1997**, *45*, 4635–4638.
37. Hendrich, S.; Lee, K.-W.; Xu, X.; Wang, H.-J.; Murphy, P. A. Defining food components as new nutrients. *J. Nutr.* **1994**, *124*, 1789S–1792S.
38. Anderson, J. J. B.; Garner, S. C. The effects of phytoestrogens on bone. *Nutr. Res.* **1997**, *17*, 1617–1632.

Downloaded by DUKE UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch012

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Chapter 13

Antioxidant Capabilities of Defatted Soy Flour Extracts

Xiaohua Yue and Zhimin Xu*

**Department of Food Science, Louisiana State University,
Baton Rouge, LA 70803
*zxu@agctr.lsu.edu**

Antioxidant activities of defatted soy flour extract and the extract treated with heating or β-glucosidase hydrolysis were evaluated using a menhaden oil oxidation model. The defatted soy flour extract (5 % in oil) demonstrated the capability of reducing menhaden oil oxidation products and retaining over 60% of DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) after the oil was heated at 150°C for 30 min, while only 30% of DHA and EPA in the control oil model. The level of rancid volatile, hexanal, in the menhaden oil mixed with the extract was significantly reduced during storage at room temperature as well. The isoflavones with glucoside in the extract were significantly converted to their corresponding aglycone isoflavones at 150°C for 30 min with less thermal degradation or by β-glucosidase hydrolysis. The antioxidant activities of the heated and enzyme treated extracts were significantly higher than the original extract. After 4 days storage at room temperature the retained EPA and DHA in the menhaden oil were over 60% with the enzyme treated extract and 30% with the heat treated extract, while approximately 15% were remained in the fish oil with the original extract.

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Introduction

Lately, the health benefits of soy foods, such as, reducing the formation and progression of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer's disease, and osteoporosis have been reported in numerous studies and well recognized by the FDA (*1–3*). It is suggested that soy isoflavones play an important role of the health benefits (*4*). The isoflavones are effective antioxidants because of their phenol structure and redox potential (*5*). They are not lipid soluble and largely remained in defatted soy flour which is the main byproduct in soybean oil processing after soy oil in whole soy flour is extracted. The defatted soy flour could be an economical source to produce soy isoflavones concentrate and used in various health promoting foods. As the antioxidant potential of soy isoflavones, the defatted soy flour extract could be used as a natural food antioxidant to replace synthetic antioxidants in extending food shelf-life as well.

In this study, menhaden oil was used as a model to evaluate the antioxidant capability of defatted soy flour extract. Menhaden oil contains higher level of omega-3 long chain polyunsaturated fatty acid (PUFA). Benefits of daily intake of) PUFA from fish oil in preventing cardiovascular diseases have been confirmed by a number of epidemiological and clinical studies (*6–8*). However, the PUFA in fish oil are readily oxidized when exposed to light, oxygen, prooxidants and high temperatures to produce off- or rancid- flavor volatiles (*9*). Thus, the quality of fish oil or foods fortified with fish oil usually deteriorates rapidly without stabilization by antioxidants. Synthetic antioxidants, such as TBHQ (tertiary butyl hydroquinone), BHA (butylated hydroanisole) and BHT (butylated hydroxytoluene), and alpha-tocopherol acetate are used for retarding the fish oil oxidation. However, potential toxicity and mutagenicity of these artificial chemicals have been concerned for many years (*10*). BHA could convert ingested material into toxic substance or carcinogens due to increased secretion of microsomal enzymes of liver and extra-hepatic organs, such as the lungs and gastrointestinal tract mucosa (*11*). Kotsonis et al. (*12*) have suggested that even small amounts of artificial antioxidants could have potentially harmful health effects from long-term consumption. Thus, the study of antioxidant capability of defatted soy flour in menhaden oil model could provide useful information of developing natural soy antioxidant used in various food lipid systems. The effectiveness level of defatted soy flour extracts in preventing PUFA oxidation and reducing the oxidation products in the fish oil model also reflected their antioxidant capability.

As antioxidant activity of a phenolic compound relies on the number of hydroxyl group on benzene ring. The more the hydroxyl group, the higher the antioxidant activity would be. Aglycone forms of isoflavones may have higher antioxidant capability than their glucoside forms of isoflavones due to the increase of hydroxyl groups. Heat treatment or hydrolysis could break down isoflavone glucosides and convert them into corresponding aglycones (Figure 1) (*13*). For instance, glucoside forms daidzin and genistin released free aglycone isoflavones under acidic conditions (*14*). Chien et al. (*15*) found the glucoside conjugated isoflavones converted to aglycone forms of isoflavones during dry heating. Also, the linkage of glucoside and aglycone of a glucoside form isoflavone could be effectively broken down by glucosidase to release glucose and aglycone. In this study, the heat and enzyme treatments were used to convert glucoside forms of isoflavones in the defatted soy flour extract to their aglycone forms of isoflavones. The change of antioxidant capability of the extract after the conversion was evaluated as well. In general, the results of this study would be helpful in utilization of defatted soy flour as a natural antioxidant in stabilizing fish oil and other food lipids.

Materials and Methods

Materials

Hexane, methanol, and butanol were HPLC grade and purchased from Fisher Scientific Inc. (Fair Lawn, NJ). BCl_3 - methanol and 2,2 - dimethoxypropane were purchased from Supelco (Bellefonte, PA). Menhaden fish oil (without any stabilizers), DPPH (2,2′—diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent, catechin, and 2-thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, Mo). Heptadecanoic acid (C17:0), docosahexaenoic acid C22:6 (DHA), eicosapentaenoic acid C20:5 (EPA), soy isoflavones (daidzin, glycitin, and genistin), and alpha and gamma tocopherol standards were from Sigma-Aldrich (St. Louis, Mo). Soybeans were purchased from a local market.

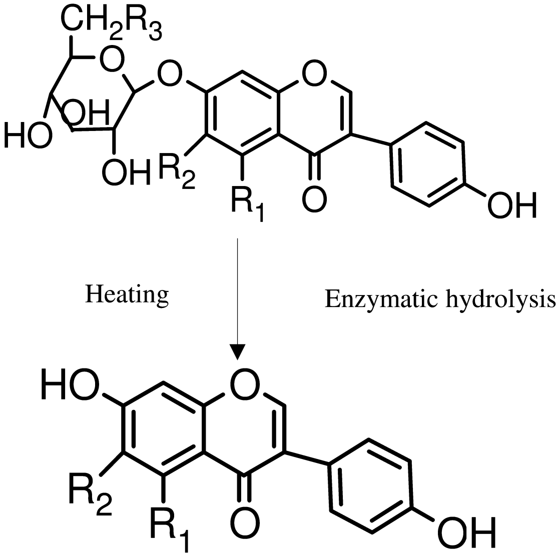


Figure 1. Structure conversion of glucoside isoflavone to aglycone isoflavone

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Preparation of Defatted Soy Flour Extract

Soybean seeds were ground and then passed through a standard 20-mesh sieve to obtain soy flour. The soy flour was mixed thoroughly and stored at -20 °C until used. Soxhlet extraction was carried out to remove lipids from soy flour. Soy flour (150 g ± 1 g) was placed in a Whatman cellulose extraction thimble (60 mm internal diameter and 180 mm external length (Whatman International Ltd, Maidston England) with a filter paper on the top of the flour. Approximately 500 ml of hexane were added into the Soxhlet apparatus. The soy flour was extracted for 8 h to remove lipids. Crude soy oil was obtained after hexane was evaporated. The defatted soy flour was taken out and spread on an aluminum foil to dry under a hood. After it was dried, the defatted soy flour was put back in the extraction thimbles and placed into the extraction apparatus again. Methanol was used as extraction solvent to extract the soy defatted flour for 8 h at 65°C. Then, the extract solvent was evaporated under a vacuum rotator evaporator (Yamato, RE 500 Yamato Scientific America, Inc., Santa Clara, CA). The defatted soy flour extract was obtained after the solvent was completely evaporated.

Preparation of Heat or Enzyme Treated Defatted Soy Flour Extract

The defatted soy flour extract was transferred into a clean beaker. The beaker was then put into a 150°C sand bath for 30 min. Heat treated defatted soy flour extract was obtained after it was cooled down at room temperature. The heat treated extract was prepared twice and mixed together and stored at -20 °C.

Twenty mg of β-glucosidase was dissolved in 50 ml buffer solution (pH=4.0, 69 ml 0.1M sodium citrate and 131 ml 0.1M citric acid) to be the enzyme solution. One gram of the defatted soy extract was weighed into a clean round flask and mixed with 10 ml of the enzyme solution. The reaction mixture was incubated at 37°C for 24 h. Then, the mixture was extracted using ethyl acetate. The solvent layer was dried by a vacuum evaporator to obtain the enzyme treated soy defatted extract. The enzyme treated extract was prepared twice and mixed together and stored at -20 °C as well.

Preparation of Fish Oil Samples

Menhaden fish oil solution was prepared by dissolving 1g oil in 100 mL of hexane. Defatted soy flour extract or the extract treated by the heat or enzyme was dissolved in methanol. An aliquot of menhaden fish oil solution was added to each test tube (13 ×100 mm). The same aliquot of the extract solution (0.5 mg /mL) was mixed with the fish oil solution by vortexing for 1 min. Then, all solutions in the test tubes were evaporated at 30°C by a centrifuge vacuum evaporator (CentriVap Mobile System; Labconco, Kansas City, MO). Thus, the dried samples were the fish oil mixed with the defatted soy flour extract and the treated extracts. The dried samples from only menhaden oil without any additive served as the control.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Fish Oil Model with Accelerated Oxidation by Heating

Twelve fish oil samples from the defatted soy flour extract and control were prepared. Three of them were used for obtaining an average initial TBA value and another three for measuring initial DHA and EPA concentrations before heating. The remaining six samples were heated at 150°C in a sand bath for 30 min. Then, three of them were used for determining their final TBA value and another three for measuring their final DHA and EPA concentrations after heating.

Determination of Total Phenolic Content

The total phenolic contents of defatted soy flour extract were determined using the Folin–Ciocalteu reagent (*16*). The Folin–Ciocalteu reagent was diluted 10 times with deionized water. The defatted soy flour extract (50 mg) were dissolved in 10 mL methanol, and 0.1 mL of this solution was mixed with 0.75 mL diluted Folin–Ciocalteu reagent. The reaction solution was left at 25 °C for 5 min. Then 0.75 mL of sodium bicarbonate solution (60 g/L) was added. The mixture was incubated at 25 °C for 90 min and filtered through a 0.45-μm syringe filter (Pall Corp., Ann Arbor, Mich., U.S.A.). The absorbance of the solution was determined at 750 nm. Catechin was used to prepare a standard curve. The total phenolic compound content was expressed as μg of catechin equivalent/g of the extract.

Determination of Isoflavone Content Using HPLC

The soy extract solution (50 mg in 10 mL methanol) was transferred to HPLC vials. The HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (id 3 mm x 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium 32 chromatography manager. The mobile phase was a mixture of water and ethanol, with percentage of water in ethanol ramped from 90% to 50% in 40 min with a constant flow rate of 0.3 mL/min (*17*). The chromatograms obtained at a wavelength of 254 nm were used to quantify the isoflavones. The concentration of each isoflavone was calculated based on their standard curves.

Determination of DHA and EPA Changes Using GC Method

The DHA and EPA were determined using the method of Li and Watkins (*18*). The sample was mixed with heptadecanoic acid (C17:0) (0.1 mg/mL in hexane as an internal standard). After adding 2 mL BCl_3-methanol and 1 mL 2, 2′-dimethoxypropane, all test tubes were capped and incubated at 60°C in a water bath for 10 min to perform the derivatization of fatty acid methyl esters. Then, 2 mL hexane and 1 mL water were added to the tubes and vortexed for 30 seconds. The upper hexane layer was transferred to another tube, dried with anhydrous sodium sulfate and transferred to a GC vial.

A gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA) with a FID detector was used to determine the DHA and EPA concentration. Helium was used as a carrier gas with a column flow rate of 1.2

mL/min. The injection volume was 5 ul and the split ratio was 1:100. The injector and detector temperature was 250 and 270°C, respectively. The oven temperature program was set to hold at 50°C for 3 min and then increased at 4.0°C/min to 250°C. The column was a Supelco SP2380 (30m × 0.25mm) (Bellefonte, PA). The concentrations of DHA and EPA were calculated using the C17:0 internal standard as a reference. The percentage of retained DHA or EPA in the fish oil after heating was obtained by comparing its final concentration in each sample to its corresponding average initial concentration.

Determination of Fish Oil Oxidation Using TBA Method

The sample was dissolved in 4 mL butanol and mixed thoroughly. Four milliliter of TBA (0.2% in butanol) solution was added to the tubes. The tubes were capped and vortexed for 1 min. The TBA reaction was carried out at 95°C in a water bath for 1 hr. After the reaction, the tubes were cooled down in ice water. The absorbance of each solution was measured at 523 nm using the UV-Visible SpectraMax Plus384 spectrophotometer. Each fish oil sample oxidation after heating was expressed by the increase of TBA absorbance value, which was the difference of the final TBA absorbance value from each heated sample and its corresponding average initial TBA absorbance value before heating.

Determination of Headspace Hexanal

The samples were placed into 2-ml vials. The vials were capped and stored at room temperature. Headspace volatile sample was taken using a syringe at day 0, 2, 4, 6, 8 and 10.

A Varian CP-3800 gas chromatography was used for the headspace volatiles analysis. The GC column was a high polarity supelcowax 10 fused silica capillary column (30m x 0.32 internal diameter, 0.1 um film) and helium was the carrier gas. Initial oven temperature was set at 60°C for 5 min, raised to 110°C at 5°C/min and maintained for 5 min. The mass detector temperature was set at 200 °C. Hexanal was identified by comparing their mass spectra and retention time with the standard and quantified using total ion peak area. The antioxidant capability of the extract was expressed by the inhibition rate, which was calculated by (1- the ratio of the peak area of treatment sample/ the peak area of the control sample) x 100.

Statistical Analysis

The means and standard deviations were calculated and the data were analyzed by one-way ANOVA with multiple comparisons at $\alpha = 0.05$ by using SAS Statistical Analysis System (SAS Inst., Cary, NC).

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Results and Discussion

Capabilities of the Defatted Soy Flour Extract in Preventing DHA and EPA Oxidation in Menhaden Oil during Heating

The levels of total phenolic content and isoflavones in the defatted soy flour extract and soy crude oil were compared (*19*). The defatted soy flour extract had much higher level of phenolic compounds (11.3 ug catechin equivalent /g) and isoflavones (55 mg/g) than the crude oil. It suggested that most phenolics and isoflavones were not extracted by hexane and remained in defatted soy flour. A similar phenomenon was observed in the study by Sun et al. (*20*), in which the total phenolic content in oat methanol extract was over three times higher than the extract produced by hexane. Compared to phenolics and isoflavones, tocopherols were less polar and largely extracted by non-polar solvent hexane. Higher tocopherol content in the extract by using hexane than acetone or methanol was reported by several studies (*20*, *21*). Thus more hydrophilic antioxidants may be highly concentrated in the defatted soy flour extract.

The capabilities of the defatted soy flour extract in preventing DHA and EPA oxidation in the fish oil model during heating are shown in Figure 2. The initial contents of EPA and DHA in menhaden oil were 13.5 % and 11.5 %, respectively. Significantly higher retained DHA and EPA were found in the fish oils mixed with the defatted soy flour extract. In the control group, only 29.9 % of DHA and 37.2 % of EPA in the fish oil were retained after being heated at 150°C for 30 min, while 62.8 % DHA and 67.7 % EPA were retained in the fish oils mixed with the defatted soy flour extract.

Meanwhile, the significantly higher antioxidant capability of defatted soy flour extract in stabilizing fish oil was also confirmed by the lower increase of TBA reactive oxidation products (Figure 3). The TBA reactive substances in fish oil were reported to increase 3 to 4 times at 4°C and 2 to 3 times at -18°C during 30 days of storage (*22*). The level of the polyunsaturated fatty acids dropped to 1/3 of the original level after 7-day storage at 25°C (*23*). In this study, the increase of TBA value in the fish oil mixed with the defatted soy flour extract was lower than the control group, although the fish oil oxidation was accelerated by heating at 150°C. The higher level of phenolics and isoflavones in the defatted soy flour extract may have contributed to the capability of stabilizing the menhaden oil during heating. The capabilities of phenolic antioxidants in chardonnay grape and black raspberry seed flour extracts in preventing the long chain polyunsaturated fatty acids of menhaden oil oxidation were reported by Luther et al. (*24*). In their study, the oxidation of total polyunsaturated fatty acids in menhaden oil mixed with 1.5% of chardonnay grape and black raspberry seed flour was retarded after 80°C incubation for 4.5 h. Sun et al. (*20*) also found that a methanol extract of oat had greater capability in inhibiting the DHA oxidation during heating than its hexane extract with higher tocopherol content. Some studies found that an extract with higher level of phenolics could significantly improve the stability of vegetable oils at a frying temperature of 180°C and reduce peroxide values during 26 days of storage at 60°C (*25*, *26*). Although the phenolic antioxidants are most likely responsible for preventing bulk oil oxidation, the higher level of soy isoflavones in the defatted soy flour extract are the important antioxidants as well.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

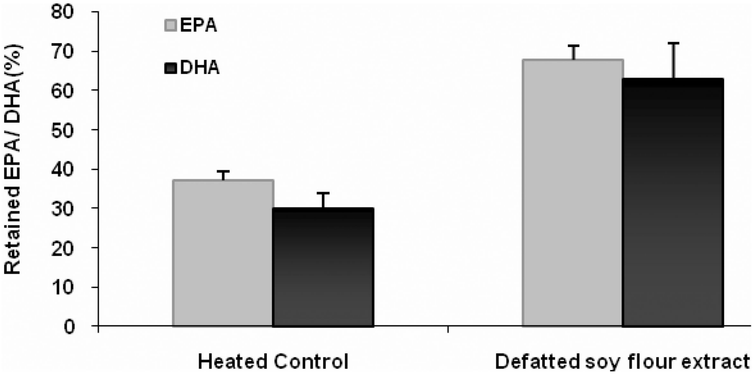


Figure 2. Retained DHA and EPA in menhaden oil mixed with the defatted soy flour extract after heating at 150°C for 30 min

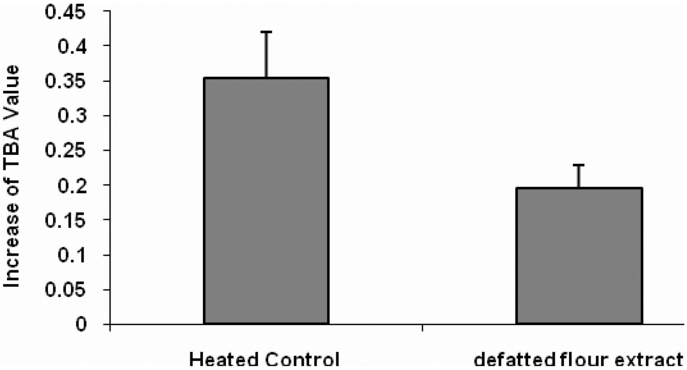


Figure 3. Changes of TBA reactive oxidation products in menhaden oil mixed with the defatted soy flour extract after heating at 150°C for 30 min

Production of Rancid Volatile in Fish Oil Mixed with or without the Defatted Soy Flour Extract during Room Temperature Storage

Undesirable odors, such as aldehydes, ketones and alcohols were produced due to the degradation of unsaturated fatty acids during storage (*25*). Hexanal is the major rancid volatile related to the lipid oxidation of fish oil. The level of the volatile reflects the development of the degree of the lipid oxidation (*27*). Compared with the control group, the production of hexanal in fish oil mixed with the defatted soy extract was significantly reduced after 4 days storage at room temperature (Figure 4). The inhibition rates of hexanal formation were about 86.7% and 65.6% after 8 and 10 days, respectively. Wettasinghe and Shahidi (*28*) reported that defatted borage seeds extract (200 ppm) could reduce the formation of hexanal in bulk corn oil after 7 days when stored at 4°C. Jayathilakan et al. (*27*) reported that cinnamon (250 mg/100g) could inhibit about 40% hexanal formation when added in beef sample during storage at 5°C. Our results indicated that the defatted soy flour extract (5% in oil) could greatly reduce the production of rancid volatiles in fish oil during storage at room temperature.

Conversion of Glucoside Isoflavones to Aglycone Isoflavones during Dry Heating

It was found that the conversion of glucoside isoflavones to their corresponding aglycone isoflavones occurred at a heating temperature at or above 150°C in this study. After 10 min at 150°C, daidzein, glycitein and genistein were significantly produced while daidzin, glycitin, and genistin were decreased (Table 1). The decrease rate from low to high was daidzin, genistin, and glycitin. The increase rate from high to low was glycitein, genistein, and daidzein (Table 1). After 30 min, the conversion of glycitin to glycitein was the highest compared with daidzin to daidzein or genistin to genistein. The results indicated that some thermal degradation of daidzin and genistin may be not take place in the linkage of their aglycone and glucoside parts and result in other not aglycone degradation products. For glycitin, the thermal degradation could preferably occur at the glucoside linkage to produce glycitein before the other structure was broken. However, when heated at temperature 200°C, the glucoside isoflavones decreased rapidly (Table 2). There was less than 5% of daidzin, glycitin and genistin remained after 10 min. Daidzein, glycitein and genistein were produced to levels of 19.1, 48.8, 9.3 ug after 10 min (Table 2). Eventually, the levels of daidzein, glycitein and genistein were 18.3, 42.8 and 7.0 ug, respectively after 30 min at 200°C. The conversion yield of glycitin to glycitein was higher than either of daidzin or genistin, which was the same as that at 150°C. Ishihara (*13*) reported the aglycone isoflavones were more bioactive than the glucoside isoflavones since they were readily absorbed across the enterocyte of the intestine (*13*). Kim and Lee (*29*) reported the antioxidant capacity level of isoflavones decreased as follows: genistein > daidzein > genistin > daidzin by using vitamin C equivalent antioxidant capacity.

The generation of aglycone from the acetyl- and malonly- glucoside form during heating was observed in several previous studies (*15*, *16*, *30*). Some studies also found that glucoside isoflavones could be produced from the degradation of malonyl forms of isoflavones (*31*, *32*) as well. Mathias et al. (*33*) and Barnes et al. (*34*) indicated in general, isoflavones were not destroyed under normal thermal processing conditions but rather were subjected to interconversions between the different forms. However, some aglycones (especially daidzein) were thermally degradable (*35*, *36*). Chien et al. (*15*) reported that genistein was generated at a slow rate after genistin was heated at 200°C. This was different from our results in which the rate was much higher than in their study. The reason might be that the degradation contributed from oxygen oxidation was eliminated due to genistin was in methanol solution flushed with nitrogen and sealed in the study of Chien et al. (*15*). As the oxidation is a very important reaction and not avoidable for most soy food processing, our degradation study was preformed in an open air environment which included the oxidation degradation during heating.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

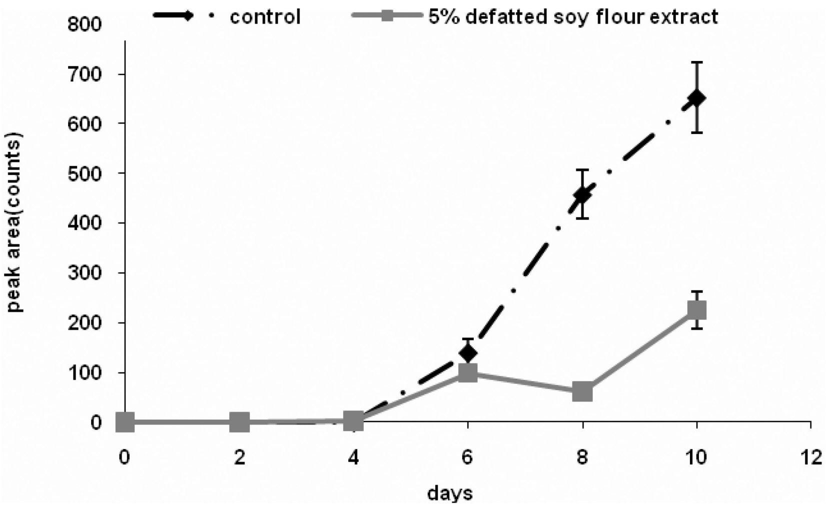


Figure 4. Hexanal production in menhaden oil and the oil mixed with the defatted soy flour extract during room temperature storage

Table 1. The content changes of glucoside and aglycone isoflavones at 150°C

Isoflavone(ug)	*time (min)*			
	0	*10*	*20*	*30*
daidzin	100.0	96.7±2.5	82.3±1.4	75.5±0.9
glycitin	100.0	70.4±2.2	46.9±1.2	29.6±1.7
genistin	100.0	87.5±2.4	77.4±3.4	69.7±0.4
daidzein	0	3.0±0.6	3.1±0.1	5.3±1.6
glycitein	0	30.1±6.7	55.4±7.9	67.1±2.4
genistein	0	4.9±0.7	4.6±1.2	6.7±1.9

Conversion of Glucoside Isoflavones to Aglycone Isoflavones Using Enzymatic Hydrolysis

The thermal liability of aglycone isoflavones during heat treatment led us to use β-glucosidase to break down glucoside group and convert glucoside isoflavones to aglycone glucoside. β-Glucosidase extracted from Aspergillus niger (β -glucoside glucohydrolase, EC 3.2.1.21) is an enzyme that is able to cleave the β-glucosidic linkages of di- and/or oligo- saccharides or other glucose conjugates. β–Glucosidases plays pivotal roles in many biological processes, such as the degradation of cellulosic biomass, cyanogenesis and the cleavage of glucosylated flavonoids (*37*). It was also reported to be able to break down glucoside groups from glucoside isoflavones to form aglycones isoflavones (*38*).

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Table 2. The content changes of glucoside and aglycone isoflavones at 200°C

Isoflavone (ug)	*time (min)*			
	0	*10*	*20*	*30*
daidzin	100.0	2.4±0.3	1.9±0.1	2.2±0.5
glycitin	100.0	3.6±0.7	2.9±0.2	2.5±0
genistin	100.0	2.2±0.4	1.8±0.2	1.5±0.2
daidzein	0	19.1±0.4	16.3±0.8	18.3±1.6
glycitein	0	48.8±0.2	43.0±1.1	42.8±2.2
genistein	0	9.3±1.3	6.6±0.2	7.0±0

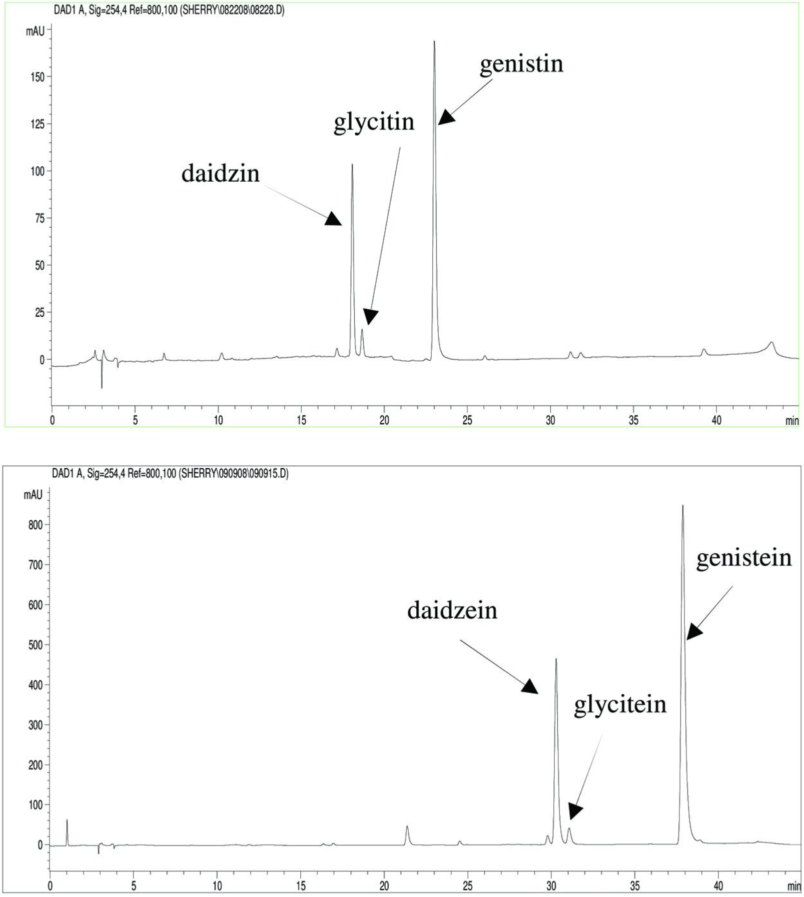


Figure 5. Chromatograms of isoflavones in the defatted soy flour extract before (above) and after (below) the enzymatic hydrolysis

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Table 3. The percentage of each isoflavone in total isoflavones content in defatted soy flour extract before and after the enzymatic hydrolysis

Isoflavones	*Glucoside form*			*Aglycone form*		
	daidzin	*glyctin*	*genistin*	*daidzein*	*glyctein*	*genis-tein*
Original extract	35.8±1.5	7.5±0.4	51.3±0.6	1.9±0.9	1.3±0.2	2.2±0.9
Enzyme treated extract	4.8±0.2	1.3±0.2	3.8±0.3	34.0±3.0	4.5±0.4	51.6±0.8

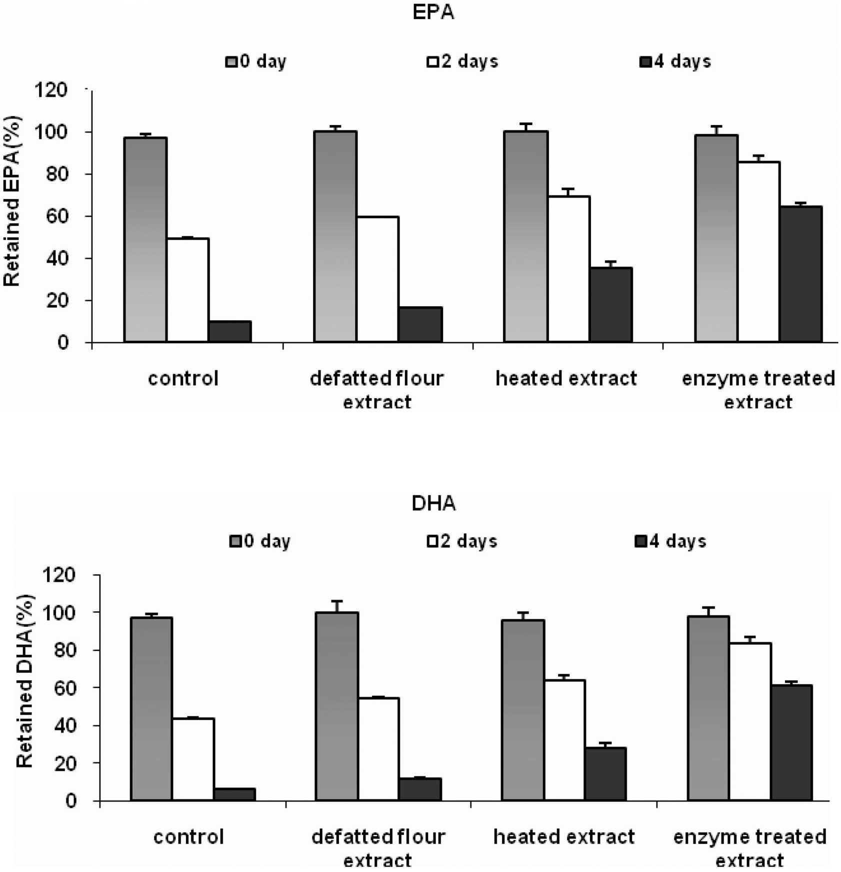


Figure 6. Retained DHA and EPA in menhaden oil mixed with the defatted soy flour extract and the extracts with the heat and enzyme treatment

Compared with the heat treatment, the conversion of glucoside isoflavone to aglycone isoflavone using glucosidase hydrolysis was more easily controlled and optimized. After the enzyme treatment, the glucoside isoflavones in the extract were greatly converted to be aglycone isoflavones. Figure 5 shows that isoflavones profile in the defatted soy flour extract before and after the enzyme treatment. In the enzyme treated defatted soy flour extract, daidzein and genistein are the major isoflavones. In the same concentration of defatted soy extract and enzyme treated extract, before the enzyme treatment, the percentages of glucoside and aglycone isoflavones of total isoflavones in defatted soy extract were 94.6 % and 5.4 %, respectively (Table 3). After the treatment, the percentage of glucoside isoflavones decreased to 9.9 %, while the percentage of aglycone isoflavones was increased to 90.1 % (Table 3).

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Capabilities of the Heat and Enzyme Treated Defatted Soy Flour Extracts in Preventing DHA and EPA Oxidation in Menhaden Oil during Room Temperature Storage

Figure 6 shows that EPA and DHA in control menhaden oil were not stable and decreased below 50% in 2 days and 10% in 4 days storage at room temperature. Both EPA and DHA in the menhaden oil mixed with the defatted soy extract were still over 50% and 10% after 2 and 4 days storage, respectively (Figure 6). However, the remained EPA and DHA were over 75% in the menhaden oil mixed with the heat treated defatted soy extract after 2 days storage. After 4 days storage, the retained EPA and DHA in the menhaden oil with the heat treated defatted soy extract were 35.5% and 28.1% respectively and twice times higher than that in the menhaden oil mixed with the original defatted soy extract. These findings suggest that thermal treatment could effectively improve the antioxidant activity of defatted soy flour extract. The reason might be that the higher level of aglycone isoflavones in the heated defatted soy extract could enhance the antioxidant activity since they have an additional –OH group on the benzene ring.

The capability of inhibiting EPA and DHA oxidation of defatted soy flour extract was significantly enhanced after the enzyme treatment as well. Both EPA and DHA in the menhaden oil mixed with the enzyme treated defatted soy extract remained over 80% in 2 days and 60% in 4 days during room temperature storage. The retained levels were five times and twice higher than those in the control oil and menhaden oil mixed with the heat treated extract, respectively. These results supported the study of Naim et al. (*39*) who reported that aglycone isoflavones had greater antioxidant activities than their glucoside isoflavones. Also, Rimbach et al. (*40*) reported that aglycone isoflavones had higher antioxidant ability than their sulfate conjugated forms. Ishihara (*13*) also found that the genistein had the highest antioxidant activity followed by daidzein series, aglycone conjugates and glucosidic conjugates using the ABTS (2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) radical reaction assay.

In conclusion, the defatted soy flour extract showed the capability for stabilizing menhaden oil and preventing DHA and EPA oxidation during heating or storage at room temperature. It suggests that the extract could significantly retard food lipid oxidation during cooking or storage. It also prevented the production of undesirable and toxic lipid oxidation products. The antioxidant activity of the extract can be enhanced after the extract was heated at 150°C for 30 min or hydrolyzed by β-glucosidase. The enzyme treated defatted soy flour extract had the higher antioxidant activity in inhibiting the PUFA oxidation in menhaden oil. Thus, in addition to the health promoting function, the defatted soy flour extract with higher level of isoflavones could be utilized as an antioxidant ingredient to stabilize food lipids and extend their shelf-life.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

References

1. Martinez-Tome, M.; Murcia, M. A.; Frege, N.; Ruggieri, S.; Jimenez, A. M.; Rose, F.; Parras, P. *J. Agric. Food Chem.* **2004**, *52*, 4690.
2. Nyström, L.; Mäkinen, M.; Lampi, A. M.; Piironen, V. *J. Agric. Food Chem.* **2005**, *53*, 2503.
3. Truswell, A. *Eur. J. Clin. Nutr.* **2002**, *56*, 1.
4. Messina, M. J. *Am. J. Clin. Nutr.* **1999**, *70*, 439S.
5. Meng, Q. H.; Lewis, P.; Wahala, W.; Adlercreutz, H.; Tikkanen, M. J. *Biochem. Biophys. Acta* **1999**, *1438*, 369.
6. Crombie, I. K.; McLoone, P. I.; Smith, W. C.; Thomson, M.; Pedoe, H. T. *Eur. Heart J.* **1987**, *8*, 560.
7. Dyerberg, J.; Bang, H. O.; Stoffersen, E.; Moncada, S.; Vane, J. R. *Lancet* **1978**, *312*, 117.
8. Wang, C.; Harris, W. S.; Chung, M.; Lichtenstein, A. H.; Balk, E. M.; Kupelnick, B.; Jordan, H. S.; Lau, J. *Am. J. Clin. Nutr.* **2006**, *84*, 5.
9. McClements, D. J.; Decker, E. A. *J. Food Sci.* **2000**, *65*, 1270.
10. Wanasundara, U. N.; Shahidi, F. *Food Chem.* **1998**, *63*, 335.
11. Wattenberg, L. W. *Food Chem. Toxicol.* **1986**, *24*, 1099.
12. Kotsonis, F. N.; Burdock, G. A.; Flamm, W. G. Food Toxicology. In *Casarett and Doull's Toxicology*; McGraw-Hill Co.: New York, 2001; p 1049.
13. Ishihara, M. *J. Sci. Food Agric.* **2007**, *87*, 2844.
14. Wang, G.; Kuan, S. S.; Francis, O. J.; Ware, G. M.; Carman, A. S. *J. Agric. Food Chem.* **1990**, *38*, 185–190.
15. Chien, J. T.; Hsieh, H. C.; Kao, T. H.; Chen, B. H. *Food Chem.* **2005**, *91*, 425.
16. Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. *J. Agric. Food Chem.* **1998**, *46*, 4113.
17. Xu, Z.; Wu, Q.; Godber, S. *J. Agric. Food Chem.* **2002**, *50*, 7402.
18. Analysis of Fatty Acids in Food Lipids. In *Current Protocols in Food Analytical Chemistry*; Li, Y., Watkins, B. A., Eds.; John Wiley & Sons, Inc.: New York, 2002, p D1.2.1
19. Yue, X.; Xu, Z.; Prinyawtwatkul, W.; Losso, J. N.; King, J.; Godber, J. S. *J. Food Sci.* **2008**, c19.
20. Sun, T.; Xu, Z.; Godber, J. S.; Prinyawiwatkul, W. *Cereal Chem.* **2006**, *83*, 451.
21. Moreau, R. A.; Powell, M. J.; Singh, V. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1063.

22. Boran, G. *Food Chem.* **2006**, *98*, 693.
23. Borquez, R.; Koller, W.; Wolf, W.; Spiess, W. E. L. *Lebensm.−Wiss. Technol.* **1997a**, *30*, 502.
24. Luther, M.; Parry, J.; Moore, J.; Meng, J.; Zhang, Y.; Cheng, Z.; Yu, L. *Food Chem.* **2007**, *104*, 1065.
25. Duve, K. J.; White, P. J. *J. Am. Oil Chem. Soc.* **1991**, *68*, 365.
26. Tian, L. L.; White, P. J. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1079.
27. Jayathilakan, K.; Sharma, G. K.; Radhakrishna, K.; Bawa, A. S. *Food Chem.* **2007**, *105*, 908.
28. Wettasinghe, M.; Shahidi, F. *Food Chem.* **1999**, *67*, 399.
29. Kim, D.; Lee, C. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 253.
30. Rostagno, M. A.; Palma, M.; Barroso, C. G. *Food Chem.* **2005**, *93*, 557.
31. Coward, L.; Smith, M.; Kirk, M.; Barnes, S. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S.
32. Mahungu, S. M.; Diaz-Mercado, S.; Li, J.; Schwenk, M.; Singletary, K.; Faller, J. *J. Agric. Food Chem.* **1999**, *47*, 279.
33. Mathias, K.; Ismmail, B.; Corvalan, C. M.; Hayes, K. D. *J. Agric. Food Chem.* **2006**, *54*, 7495.
34. Barnes, S.; Coward, L.; Kirk, M.; Sfakjanos, J. **1998**, *217*, 254.
35. Huang, H.; Liang, H.; Kwok, K. *J. Sci. Food Agric.* **2006**, *86*, 1110.
36. Murphy, P. A.; Barua, K.; Hauck, C. C. *J. Chromatogr., B.* **2002**, *777*, 129.
37. Kuo, L. C.; Lee, K. T. *J. Agric. Food Chem.* **2008**, *56*, 119.
38. Tsangalis, D.; Ashton, J. F.; McGill, A. E.; Shah, N. P. *J. Food Sci.* **2002**, *67*, 3104.
39. Naim, M.; Gestetner, B.; Bondi, A.; Birk, Y. *J. Agric. Food Chem.* **1976**, *24*, 1174.
40. Rimbach, G.; Weinberg, P. D.; Pascual-Teresa, S.; Alonso, M. G.; Ewins, B. A.; Turner, R.; Minihanec, A. M.; Botting, N.; Fairley, B.; Matsugo, S.; Uchida, Y.; Cassidy, A. *Biochim. Biophys. Acta* **2004**, *1670*, 229.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch013

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

Chapter 14

Tofu Structure Is Regulated by Soymilk Protein Composition and Coagulant Concentration

Tomotada Ono,* Yuzuru Onodera, Yeming Chen, and Katuhiko Nakasato

**United Graduate School of Agricultural Science, Iwate University,
Ueda 3, Morioka 020-8550, Japan
*tomon@iwate-u.ac.jp**

Tofu is made from soymilk which is a turbid colloidal liquid. Tofu structure is related to soymilk components, particularly colloidal components such as protein particles and oil globules. Protein particle content increased with the increase of 11S/7S globulin ratio in soybeans. Tofu was made from the soybean mixtures having different 11S/7S ratio by adding coagulants of various concentrations. The tofu having high 11S/7S ratio showed a hard texture at low coagulant concentration, and the tofu having low 11S/7S ratio needed more coagulant for the maximum hardness. Before the tofu reached maximum hardness, the tofu structure had a large cell and thin wall of network, and after it reached the maximum hardness, its network structure showed a large cell with many holes and no flat wall. However, the tofu structure near maximum hardness had fine and even cells with the coagulant concentration of a changing point from increase of hardness to constant. Therefore, tofu from soybeans having larger 11S/7S ratios could have a fine structure with lower coagulant concentration.

Soybeans containing 30-40% protein and 20-30% lipid have been utilized for an important dietary source of protein and lipid since ancient times. The major products are soymilk and tofu, which are used in many Asian countries as milk and as cheese in western countries. Tofu is made from soymilk by the addition of a coagulant. The properties of tofu are limited by the quality of soymilk.

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Therefore, at first, soymilk formation and the second, tofu curd formation are described, and then we expressed about the relation between tofu structure and protein composition at various concentrations of coagulant.

Soymilk Formation

Soymilk is a stable turbid colloidal liquid containing protein, lipid and carbohydrates similar to cow's milk (*1*). The stable colloidal liquid is made by grinding, heating, and filtration of swelled soybeans. Soymilk contains almost all proteins and lipids and a half of carbohydrate in soybean, and is formed as follows; Proteins and lipids in soybeans are extracted by grinding with water, also oligo-sugars being extracted. Lipids globules precipitated with proteins and exist in conjugated form in raw soymilk as shown in Figure 1 (*2*). Proteins are then released from lipid globules by heating at more than 65°C and then 7S and 11S globulins are decomposed to subunits at 75°C and above 80°C, respectively (*2*), since 7S and 11S are unfolded to subunits from above 75°C and above 80°C, respectively. The protein particles having about 100nm diameter are formed from these subunits (*1*). Lipid globule in raw soymilk becomes oil body at above 75°C by releasing bound proteins. The formation mechanism of soymilk is shown in Figure 2. The major components of soymilk are oil body and soluble and particulate proteins.

There are two manufacturing processes for regular (traditional) and reconstituted (modern) soymilk. The traditional process involves a heating process after grinding of soaked soybeans. However, some people are uncomfortable with the natural bean-like (or raw grass) flavor of soymilk. In modern processing, grinding and heating are carried out simultaneously, reducing the been-like flavor, and thus increasing the popularity for this type of soymilk. In traditional process, two types of soymilk are also formed by filtration before or after heating which are called as nama-shibori (raw-squeezed) and kanetsu-shibori (heat-squeezed), respectively. These traditional soymilks are used for tofu making.

Soymilk is a stable colloid like cow's milk; although containing linoleic acid lipids are not oxidized, and protein particles having about 100nm diameter do not precipitate. The method of making soymilk is an important technique for making a stable colloidal protein and can be applied to other processing.

Tofu (Soybean Curd) Formation

Tofu is a curd-like traditional food prepared from soymilk by the addition of a coagulant and has slight taste; therefore, the sensory evaluation, particularly of the physical property, is important for the quality control of tofu. Many studies have reported the factors that influence the physical properties of tofu. These factors originate from the differences in soybean components that change depending on the varieties of soybean and cultivation conditions (*3*–*5*). It is generally recognized that the use of soybean varieties with high protein content results in the production of firm tofu. Wang and Hesseltine (*6*) and Shen *et al.* (*7*) reported that varieties with high protein content produced tofu with a firmer

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

and springier texture. Tofu manufacturers in Japan have commonly used soybean varieties (Fukuyutaka, Enrei, etc.) with high protein content. On the other hand, it is reported that the gel hardness of tofu made from purified soy proteins is positively correlated with the 11S (glycinin) content (*8–10*).

Since tofu curd is prepared from soymilk containing not only proteins but also lipids, carbohydrates, and so on by the addition of a coagulant, the factors that influence curd firmness remained to be clarified (*3*, *5*, *11*). Ono *et al.* (*12*) and Guo *et al.* (*13*) found that the main components of soymilk involved in the formation of tofu curd are particulate and soluble proteins and lipid globules. Each fraction of particulate and soluble proteins was obtained from soymilk by ultracentrifugation and dispersing into ultra-filtrate of soymilk. Protein Solubility of particulate protein decreased at lower Ca concentration than that of soluble protein as shown in Figure 3 (*12*). Solubility of soymilk proteins (not separated), however, was not decreased even at Ca concentration where all particulate protein (separated) precipitated although that in soymilk should precipitate. The separated protein solutions did not contain lipid globules. Therefore, each solubility of particulate proteins and lipid globules in the mixture was examined as shown in Figure 4a (*13*). Both of particulate proteins and floating fraction (lipid globules) decreased in the same manner from the mixture suspension. On the other hand, soluble proteins and floating fraction in the mixture decreased after new particulate proteins were formed by addition of Ca as shown in Figure 4b. From these results a new theory of tofu (curd) formation was advocated as the curd formation occurs in 2 steps: 1) conjugation of protein particles on the surface of lipid globules at the early stage of the addition of the coagulant and 2) formation of a network with non-repulsive lipid globules covered with protein particles (*14*). The curd formation mechanism is shown in Figure 5. During the tofu making, the oil body, which was originally packed with oil body protein (oleosin), was packed with protein particles and then furthermore packed with the particles formed from soluble proteins. Therefore, the lipids in tofu are very stable against oozing and oxidation by cooking and storing. This technique from a traditional processing could be able to apply to a new food making.

Tofu Structure Regulated by 11S/7S Ratio and Coagulant Concentration

Tezuka *et al.* (*15*), Guo *et al.* (*16*), and Guo and Ono (*17*) used soybeans having different subunits of 11S (glycinin) and 7S (β-conglycinin) and reported that the ratio of protein particles in soymilk is increased by increasing the 11S protein, and that the increase of the particles introduced the firm tofu (Figure 6). The firm tofu could be made by the network formation with oil globules covered with more protein particles in 11S rich soymilk. Many recent reports have shown that tofu prepared from soybeans with high 11S content had higher breaking stress than that prepared from soybeans with low 11S content (*18–22*).

On the other hand, Toda *et al.* (*23*) highlighted the importance of coagulant concentration from that tofu firmness is correlated not only with protein concentration but also with protein composition at low coagulant concentration

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

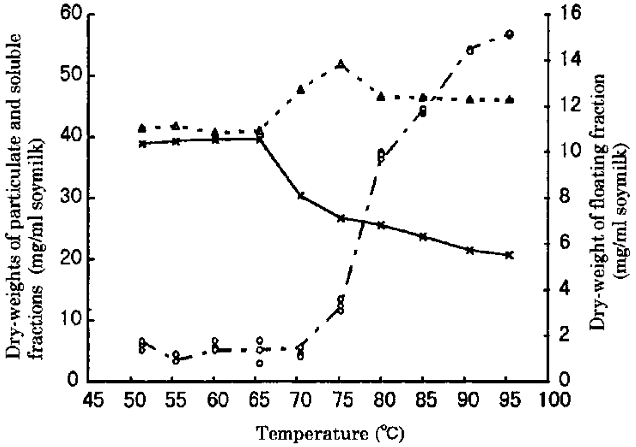


Figure 1. Dry-weight of each fraction obtained from unheated soymilk at elevated temperature (Guo et al. (2)). The soymilk was heated at a specific temperature for 5 min, cooled to 20 °C , and fractionated to particulate (x), soluble (△), and floating (○) fractions by centrifugation at 156000g for 30 min.

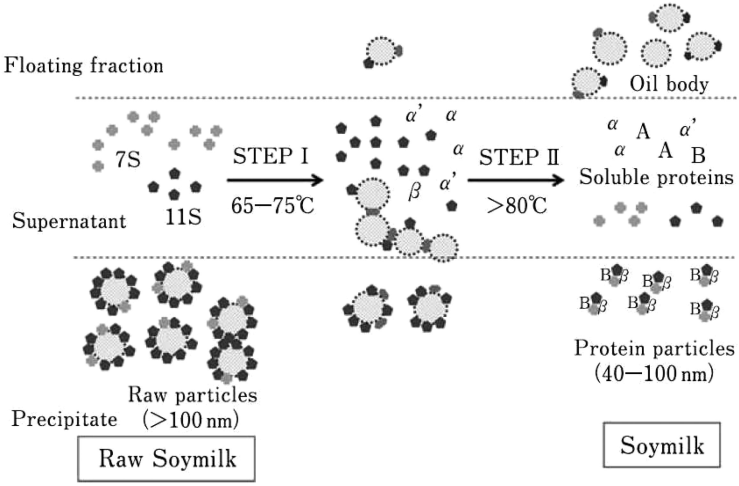

Floating fraction
Oil body
7S
11S
Supernatant
STEP I
65—75℃
α'
α
α
β
α'
STEP Ⅱ
>80℃
α
A
α'
α
A
B
Soluble proteins
Bβ
Protein particles
(40—100 nm)
Raw particles
(>100 nm)
Precipitate
Raw Soymilk
Soymilk

Figure 2. Schematic diagram of Soymilk formation from raw soymilk by heating,which was induced from Ono et al. (1) and Guo et al. (2).

and found that each soybean has a particular coagulant concentration for the maximum tofu firmness. It is already shown in Figure 3 that protein particles in soymilk aggregate at lower coagulant concentration than soluble proteins. Tofu curd from 11S-rich soymilk is formed at low coagulant concentration because 11S-rich soymilk has greater number of protein particles and tofu becomes harder with an increase in the protein particle content (Figure 6) (*17*). The relationship between coagulant concentration and protein composition should be elucidated for the optimal tofu structure. Kao *et al.* (*24*) and Liu *et al.* (*25*) reported that

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

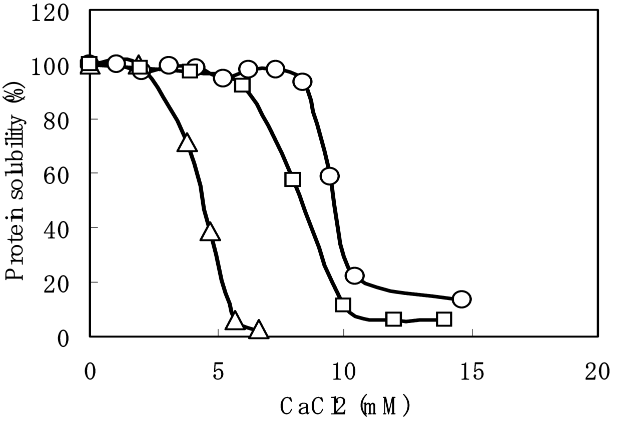


Figure 3. Effects of Calcium on Protein Solubility in Soymilk. Particulate protein (Δ) dispersed in ultrafiltrate, soluble protein (○) and soymilk (□). This Figure was prepared from Ono et al. (12).

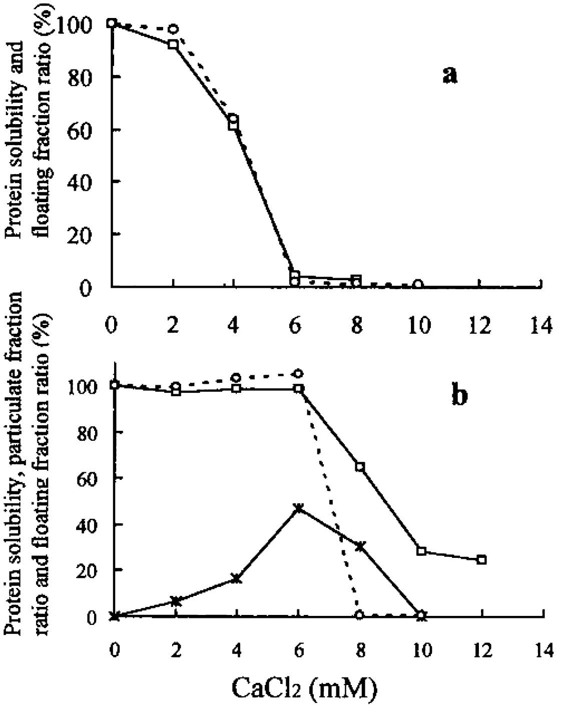


Figure 4. Changes in protein solubility and floating fraction ratio in dispersions of floating and particulate fractions (a) and floating and soluble fractions (b) at various concentrations of calcium chloride. (□) protein solubility; (○) floating fraction ratio; (✱) newly formed particulate fraction ratio. (Guo et al. (13)).

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

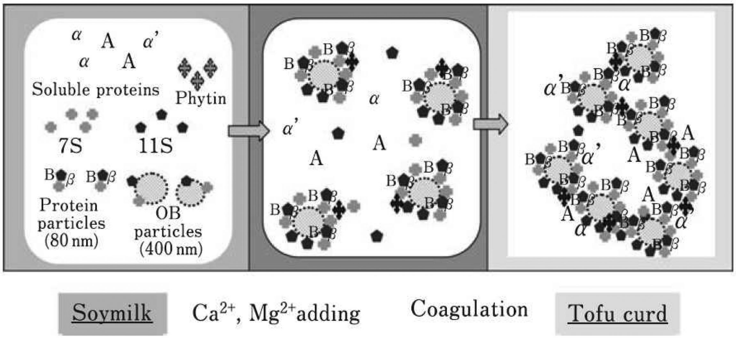


Figure 5. Formation of Tofu Curd from Soymilk (Speculation). An oil body (OB) particle is packed with oleosin proteins. (Ono (14)).

the tofu which most efficiently retained soybean proteins and water showed more homogeneous and finer microstructure in scanning electron microscopy.

Tofu were prepared from soymilks having various 11S/7S ratios by adding various concentrations of coagulant ($CaSO_4{\cdot}2H_2O$) (*26*). The breaking stress, Yang's modulus, and breaking strain values of these tofu are shown in Figure 7. The increase in the 11S content (Y/T from 0:4 to 4:0) resulted in quick attainment of the maximum of breaking stress (a) and Yang's modulus (b) values at low coagulant concentration. The breaking strain values (c) of the tofu also showed similar tendency to those of the breaking stress. These values of the tofu having higher 11S content attained a constant value at low coagulant concentration.

On the other hand, when glucono-δ-lactone (GDL) was used as a coagulant (*26*), the breaking stress and Yang's modulus values of tofu increased with increasing GDL coagulant concentration, but the extent of the increase was larger and changed at lower coagulant concentration with increasing 11S content. The breaking strain values increased up to some GDL concentration and attained a constant value at above concentration. GDL concentration of the changing point decreased gradually with increasing 11S content. The increase in 11S protein content resulted in the attainment of the maximum value at low coagulant concentration.

The formation of tofu curd by $CaSO_4$ coagulant is progressed at heterogeneous concentration system with dissolving the coagulant (suspended reagent). On the other hand, the formation of tofu curd with GDL is progressed at homogeneous concentration system after suspending and dissolving homogeneously in soymilk. When the more $CaSO_4$ coagulant was added to soymilk, the tofu should be made in the more heterogeneous concentration system and must have the more cracks in the curd. Therefore, it is considered that the breaking stress of $CaSO_4$–tofu does not increase after the network formation at the optimal coagulant concentration. Since the GDL–tofu has no cracks in the curd, the breaking stress must increase according to pH decrease at more than optimal concentration of the coagulant. The breaking strain may be due to the bonding of tofu network which is dependent on the solubility of proteins against pH. The lowest solubility region of soybean proteins has some wide range of pH (*27*). Therefore, the breaking strain values of GDL-tofu may become constant at more than the optimal coagulant concentration.

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

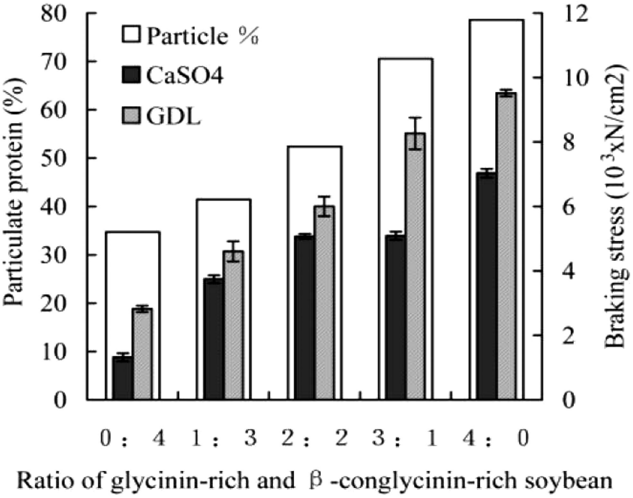


Figure 6. Comparisons of protein particle content in soymilk and the breaking stress of tofu curds prepared by mixture of glycinin-rich and β-conglycinin-rich soybeans. (Guo and Ono (17)).

The scanning electron microscopic (SEM) photographs of these tofu are shown in Figure 8 (*26*). The microstructures of tofu at lower coagulant ($CaSO_4 \cdot 2H_2O$) concentrations than the changing point of the physical property (Figure 7) showed big cells (10μm <) and flat wall as shown in Figure 8a and d, and at higher coagulant concentrations also showed larger non-uniform cells (about 5-15μm) as shown in Figure 8c, f, h, and i. Those near the changing point of physical property showed fine uniform cells (5μm>) as shown in Figure 8b, e, and g. The fine tofu was made at coagulant concentration near the changing point of the physical properties, which must show the optimal coagulant concentration for tofu structure.

The review of SEM photographs of the tofu network structure against coagulant concentrations is shown in Figure 9. At low coagulant concentration, the tofu network shows large cell-like structure, and at the changing point of tofu hardness (from increase to constant), the network shows fine uniform cells. At higher coagulant concentration, the tofu cells increase in size with a loss of smoothness of the cell wall. In the case of tofu containing more 11S protein, the change point of tofu hardness shifted to lower coagulant concentration, with a subsequent shift in the formation of network of fine uniform cells (Figures 7, 8). Therefore, the tofu network shows the uniform finest structure at the change point of tofu physical properties. Kao *et al.* (*24*) and Liu *et al.* (*25*) reported that the tofu most efficiently retaining soybean proteins and water showed more homogeneous and finer microstructure on SEM. This implies that almost all proteins in soymilk were incorporated into the tofu network at the change point of tofu breaking stress (using $CaSO_4$) or breaking strain (using GDL) and that the finer tofu network was formed by bonding of small uniform cells. The WHC of tofu decreased with increasing coagulant ($CaSO_4$) concentration (Figure 7) and was not the maximum at the change point of tofu breaking stress. The WHC of tofu should be greater at

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

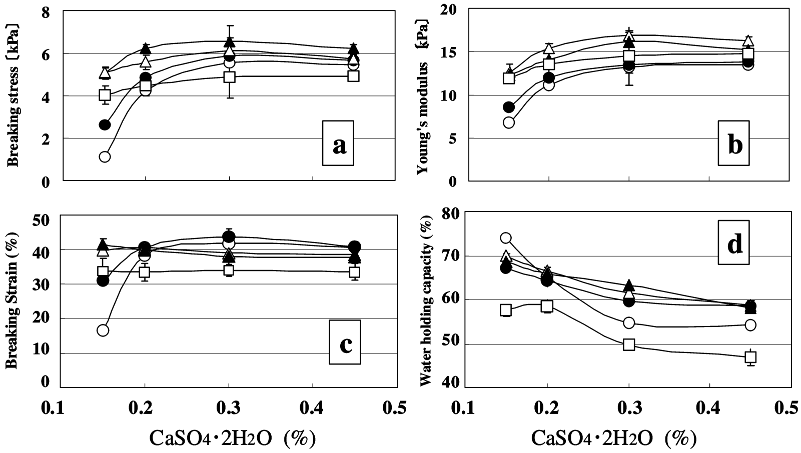


Figure 7. Changes in the textural properties of tofu prepared from adjusted soymilks against coagulant ($CaSO_4{\cdot}2H_2O$) concentration. Adjusted soymilk from soybeans with different Yumeminori (Y) :Tosan 205 (T) ratios; ○ is 0:4; ● is 1:3; △ is 2:2; ▲ is 3:1; □ is 4:0. (Onodera et al. (26)).

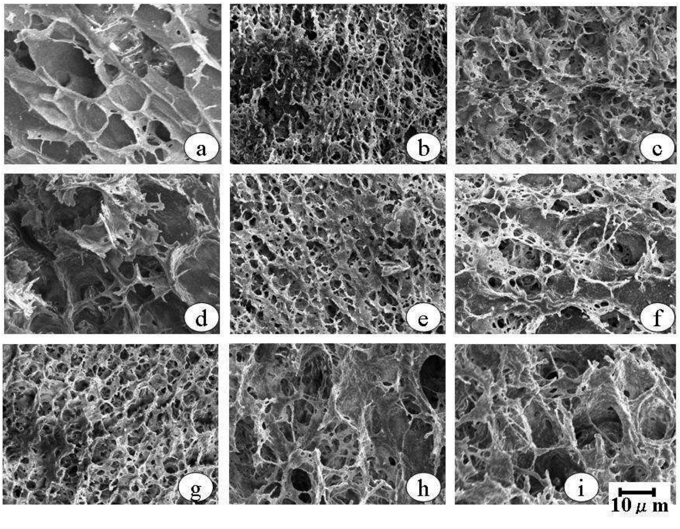


Figure 8. Scanning electron micrographs of tofu prepared from adjusted soymilks and various coagulant ($CaSO_4{\cdot}2H_2O$) concentrations. Adjusted soymilks were prepared from soybeans with different Yumeminori: Tosan 205 ratios; a,b,c are 0:4; d,e,f are 2:2; g,h,i are 4:0. Coagulant concentration: a, d, and g are 0.15%; b, e, and h are 0.3%; c, f, and i are 0.45%. (Onodera et al. (26)).

the farther range from the isoelectric point of protein because of the hydrophilic characteristic of ions. The addition of $CaSO_4$ coagulant is decreased the pH of soymilk near the isoelectric point of protein (*12*). As a result, the WHC of filled tofu could have decreased with decreasing pH.

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

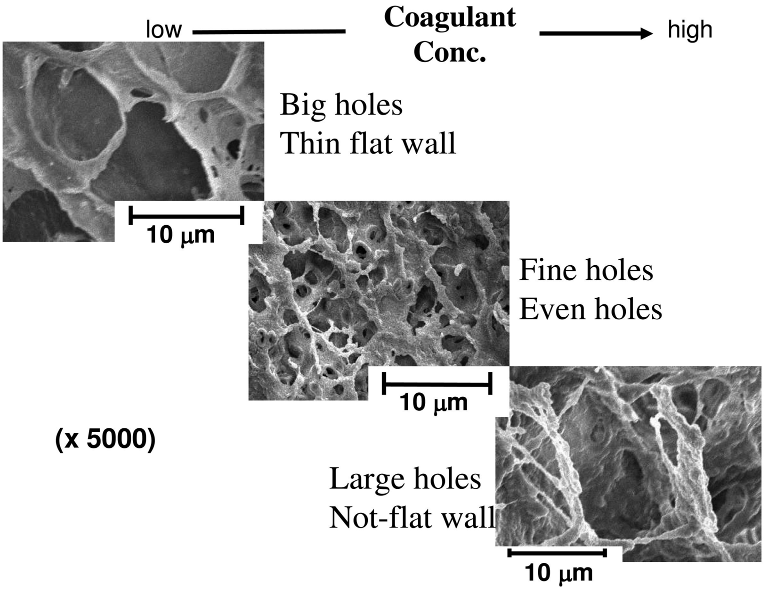


Figure 9. SEM photographs of the tofu network structure against coagulant oncentrations. Left, center and right are tofu at low, optimal, and high coagulant concentarations, respectively. (Onodera et al. (26)).

Tofu microstructure showed the finest tofu network near the change point of breaking stress ($CaSO_4$ coagulant) or breaking strain (GDL coagulant) of tofu (*26*). The coagulant concentration for obtaining the finer tofu network (optimal concentration) is the important for tofu preparation. At concentrations below and above the optimal coagulant concentration, the tofu network consisted of large cell-like units, while at the optimal concentration it consisted of the smallest and uniform units (Figure 8). The structural change point of tofu rich in 11S globulin was at lower coagulant concentration (0.15%~0.2%) and that of tofu rich in 7S was at higher coagulant concentration (0.3%~0.4%).

References

1. Ono, T.; Choi, M. R.; Ikeda, A.; Odagiri, S. Changes in the composition and size distribution of soymilk protein particles by heating. *Agric. Biol. Chem.* **1991**, *55*, 2291–2297.
2. Guo, S. T.; Ono, T.; Mikami, M. Interaction between protein and lipid in soybean milk at elevated temperature. *J. Agric. Food Chem.* **1997**, *45*, 4601–4605.
3. Skurray, G.; Cunich, J.; Carter, O. The effect of different varieties of soybean and calcium ion concentration on the quality of tofu. *Food Chem.* **1980**, *6*, 89–95.
4. Wang, H. L.; Hesseltine, C. W. Coagulation conditions in tofu processing. *Process Biochem.* **1982**, *17*, 7–12.

5. Taira, H. Quality of soybeans for processed foods in Japan. *Jpn. Agric. Res. Q.* **1990**, *24*, 224–230.
6. Wang, H. L.; Hesseltine, C. W. Coagulation conditions in tofu processing. *Process Biochem.* **1982**, *17*, 7–12.
7. Shen, C. F.; DeMan, L.; Buzzell, R. I.; DeMan, J. M. Yield and quality of tofu as affected by soybean and soymilk characteristics: Glucono-δ-lactone coagulant. *J. Food Sci.* **1991**, *56*, 109–112.
8. Saio, K.; Kamiya, M.; Watanabe, T. Food processing characteristics of soybean 11S and 7S proteins. Part I. Effect of difference of protein component among soybean varieties on formation of tofu curd. *Agric. Biol. Chem.* **1969**, *33*, 1301–1308.
9. Saio, K. Tofu: Relationships between texture and fine structure. *Cereal Foods World* **1979**, *24*, 342–354.
10. Kang, I. J.; Matsumura, Y.; Mori, T. Characteristics of texture and mechanical properties of heat-induced soy protein gels. *J. Am. Oil Chem. Soc.* **1991**, *68*, 339–345.
11. Murphy, P. A.; Chen, H. P.; Hauck, C. C.; Wilson, L. A. Soybean storage protein composition and tofu quality. *Food Technol.* **1997**, *51*, 86–88.
12. Ono, T.; Katho, S.; Mothizuki, K. Influences of calcium and pH on protein solubility in soybean milk. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 24–28.
13. Guo, S. T.; Ono, T.; Mikami, M. Incorporation of soy milk lipid into protein coagulum by addition of calcium chloride. *J. Agric. Food Chem.* **1999**, *47*, 901–905.
14. Ono, T. *The Mechanisms of Curd Formation from Soybean Milk To Make a Stable Lipid Food*, Proceedings of the 3rd International Soybean Processing and Utilization Conference, Korin, Tokyo, 2000; Kourin Publishing Co.: Tokyo, Japan, pp 51−52.
15. Tezuka, M.; Taira, H.; Igarashi, Y.; Yagasaki, K.; Ono, T. Properties of tofus and soy milks prepared from soybeans having different subunits of glycinin. *J. Agric. Food Chem.* **2000**, *48*, 1111–1117.
16. Guo, S. T.; Tsukamoto, C.; Takahasi, K.; Yagasaki, K.; Nan, Q. X.; Ono, T. Incorporation of soymilk lipid into soy protein coagulum by the addition of calcium chloride. *J. Food Sci.* **2002**, *67*, 3215–3219.
17. Guo, S. T.; Ono, T. The role of composition and content of protein particles in soymilk on tofu curding by glucono-δ-lactone or calcium sulfate. *J. Food Sci.* **2005**, *70*, C258–262.
18. Cai, T.; Chang, K. C. Processing effect on soybean storage proteins and their relationship with tofu quality. *J. Agric. Food Chem.* **1999**, *47*, 720–727.
19. Yagasaki, K.; Kousaka, F.; Kitamura, K. Potential improvement of soymilk gelation properties by using soybeans with modified protein subunit compositions. *Breed. Sci.* **2000**, *50*, 101–107.
20. Cai, R.; Mccyrdy, A.; Baik, B. K. Textural property of 6 legume curds in relation to their protein constituents. *J. Food Sci.* **2002**, *67*, 1725–1730.
21. Tay, S. L.; Perera, C. O. Physicochemical properties of 7S and 11S protein mixtures coagulated by glucono-δ-lactone. *J. Food Sci.* **2004**, *69*, 139–143.
22. Kim, Y.; Wicker, L. Soybean cultivars impact quality and function of soymilk and tofu. *J. Sci. Food Agric.* **2005**, *85*, 2514–2518.

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

23. Toda, K.; Ono, T.; Kitamura, K.; Hajika, M.; Takahashi, K.; Nakamura, Y. Seed protein content and consistency of tofu prepared with different magnesium chloride concentrations in six Japanese soybean varieties. *Breed. Sci.* **2003**, *53*, 217–223.
24. Kao, F. J.; Su, N. W.; Lee, M. H. Effect of calcium sulfate concentration in soymilk on the microstructure of firm tofu and the protein constitutions in tofu whey. *J. Agric. Food Chem.* **2003**, *51*, 6211–6216.
25. Liu, Z. S.; Chang, S. K. C.; Li, L. T.; Tatsumi, E. Effect of selective thermal denaturation of soybean proteins on soymilk viscosity and tofu's physical properties. *Food Res. Int.* **2004**, *37*, 815–822.
26. Onodera, Y.; Ono, T.; Nakasato, K.; Toda, K. Homogeneity and microstructure of tofu depend on 11S/7S globulin ratio in soymilk and coagulant concentration. *Food Sci. Technol. Res.* **2009**, *15*, 265–274.
27. Tandang, M. R. G.; Atsuta, N.; Maruyama, N.; Adachi, M.; Utsumi, S. Evaluation of the solubility and emulsifying property of soybean proglycinin and rapeseed procruciferin in relation to structure modified by protein engineering. *J. Agric. Food Chem.* **2005**, *53*, 8736–8744.

Downloaded by CORNELL UNIV on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch014

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Chapter 15

Standardization of Physical Parameters for Instron Texture Analysis for Tofu Quality Evaluation

Shaohong Yuan and Sam K. C. Chang*

Department of Cereal and Food Sciences, North Dakota State University,
Fargo, ND 58108
***Kow.chang@ndsu.edu**

Tofu is becoming a popular health food worldwide. Texture quality is the most important factor in tofu quality evaluation and trade. Commercial tofu are categorized and marketed according to firmness or hardness of tofu. However, standardized parameters for firmness analysis are lacking. Furthermore, the relationships between the instrumental and sensory quality has not been established. A valid mechanical method for textural analysis should have good correlations with human sensory data. Our objective was to develop a method for testing tofu using Instron universal machine, by characterizing the variations of tofu texture profiles as affected by physical parameters, and further correlating the mechanical data with that of a descriptive sensory method. Thirteen tofu samples with soft to extra firm market designations were chosen for the analysis of the texture profiles using various crosshead speeds and two degrees of penetration. Tofu samples were cut into a standard size from the outer and inner portion of tofu for comparison. Hardness (firmness) and springiness (elasticity) were also measured by the human sensory method. The results indicated that the level of plunger penetrations (50 and 75%) significantly affected textural values. Hardness obtained by both 50 and 70% penetrations significantly correlated with the sensory hardness. However, springiness obtained by only the 75% penetration had a significant correlation. Hardness values obtained using tofu specimens without skin correlated

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

with sensory data better than that with skin, which is produced from the pressing step in traditional tofu-making. Our findings provided a sound basis for the standardizing the physical parameters for determining tofu firmness and springiness using the Instron machine.

Introduction

Tofu consumption has a long history ever since its invention about 2,000 years ago in China. It can be made into many dishes and widely consumed in the Oriental countries (*1*). Tofu consumption in the Western countries is becoming more popular since soy proteins can protect heart disease (*2*). In trade, the texture of the tofu gel is the most important quality factor and determines tofu market classes/types, including extra firm, firm, regular, and soft, or momen (pressed), kinu (silken) and packed tofu. Specific texture of tofu is required for making into different dishes. Therefore, standardization of tofu texture becomes important to the consumers.

Soybeans are traditionally regarded as oilseeds. In the last 20-30 years, substantial research has been devoted to the understanding of the quality of various soybean cultivars for tofu making. Several researchers have reported the differences in the quality of various soybean cultivars for making tofu. Food-grade soybeans with large seed size, clear hilum, high protein and gelling quality are preferred by the tofu manufacturers. However, we have found that hilum color is not related to color of the soymilk and tofu. Uniformity of seeds is important to hydration of soybeans; but seed size does not affect the yield and textural quality of tofu (*3*). Therefore, selecting good quality soybeans has a practical importance in soybean trade since a good quality soybean cultivar would command a premium. Soybean cultivars with higher protein content have generally lower oil and total sugar content. However, protein content alone does not guarantee to result in good tofu quality. In other words, the soybean cultivars with higher protein contents may not produce tofu with desirable texture. Another major effect on tofu texture is from storage conditions. Soybeans after harvest must be stored on the farm warehouse and shipped to the manufacturing sites, at which additional storage may take place. Soybeans after improper storage may reduce tofu making ability.

In marketing soybeans for foods, the buyers and processors are interested in knowing the suitability of soybeans for making tofu because soybean quality will affect the processing procedures, tofu yield, consumer acceptability, sale and profit of the tofu products. The desire for good quality of soybeans by the processing industries has led the soybean breeders to breed specialty soybeans for tofu uses. Although small-scale laboratory methods have been used for tofu quality evaluation, a large-scale production method has been recommended due to processing effect (*4*). However, our laboratory has developed a small-scale method for making soft tofu that is correlated well with a large-scale production method (*5*). A flow diagram of the small-scale method is shown in Figure 1. Over the last thirty years, many mechanical methods have been used for measurement

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

of tofu textural quality; and that makes interlaboratory comparison of the textural qualities difficult. Therefore, standardization of the texture-testing procedures for tofu quality measurement and grading would facilitate the selection of soybean varieties for tofu making, and the marketing of tofu to the consumers. This chapter focuses on the development of texture testing procedures based our recent studies on tofu quality (*6*).

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

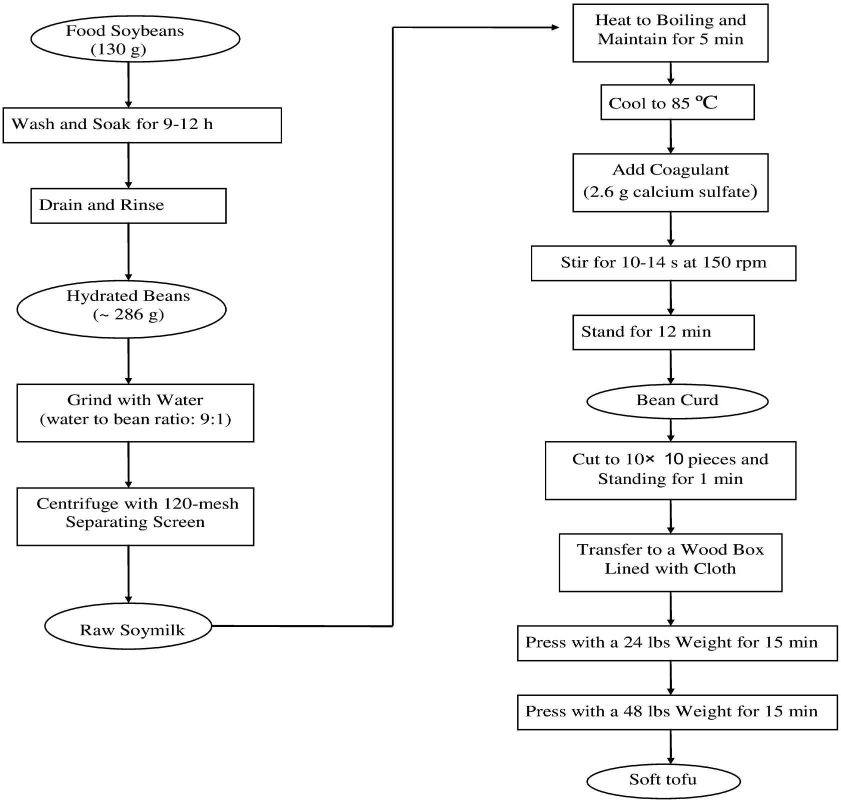

Food Soybeans (130 g)
Wash and Soak for 9-12 h
Drain and Rinse
Hydrated Beans (~ 286 g)
Grind with Water (water to bean ratio: 9:1)
Centrifuge with 120-mesh Separating Screen
Raw Soymilk
Heat to Boiling and Maintain for 5 min
Cool to 85 °C
Add Coagulant (2.6 g calcium sulfate)
Stir for 10-14 s at 150 rpm
Stand for 12 min
Bean Curd
Cut to 10× 10 pieces and Standing for 1 min
Transfer to a Wood Box Lined with Cloth
Press with a 24 lbs Weight for 15 min
Press with a 48 lbs Weight for 15 min
Soft tofu

Figure 1. A small-scale tofu making diagram

The most widely used mechanical tests for texture profile analysis (TPA) of soft and semi-soft foods were first developed by Friedman et al. (*7*) and Szczesniak et al. (*8*) using a texturometer and later modified by Bourne (*9*, *10*) using Instron universal testing machine. The characteristics of fracturability (brittleness), hardness (or firmness), cohesiveness, springiness (elasticity), adhesiveness (or stickiness), chewiness, and gumminess have been defined in the above texture profile studies (*7–10*). These studies have become very widely used in the food industries for characterization of food texture. Adhesiveness is not a significant factor in tofu products. According to Szczesniak and Bourne (*11*), chewiness is applied to solid foods only, not important to the soft tofu type, but may be more important to extra firm tofu. Fracturability is defined as the force to fracture tofu. Hardness is the force required to attain a given deformation of tofu. Cohesiveness is related to the work required to overcome the internal bonding of the tofu material. Springiness or elasticity is described as the rate at which a deformed tofu recovers to its original condition after removal of deforming force. Gumminess is calculated as the product of hardness × cohesiveness) and defined as the energy to disintegrate tofu to a state ready for swallowing (*10*).

In mechanical analysis, specimen dimensions, and specimen preparations of the heterogeneous tofu products (curd broken and pressed type) can affect the force expression values. The physical parameter settings on the analytical instrument, such as crosshead speed in terms of compression rate (mm or cm/min) and % sample deformation in terms of the level of plunger penetration into the specimen also will affect the measurement greatly (*12*). Tofu hardness is greatly influenced by processing technologies, which may or may not include a mechanical pressing procedure for expelling water from the tofu gel. The layer of skin, commonly seen in traditional firm and extra firm tofu is harder than the interior portion of the tofu gel. Therefore, the location where the test specimens are taken from a piece of tofu gel can affect its textural profile measurements. In the literature, tofu is commonly cut into cylindrical or cubic shape. However, the location of where specimens are taken from the tofu gel is rarely reported. This is an important factor that makes comparisons of inter-laboratory's results difficult.

In the literature, several types of mechanical devices have been used for textural characterization. Among the methods, the TPA method using Instron universal machine was most reported. Mechanical testing of tofu textural properties normally employs only one single set of conditions of crosshead speed and one level of plunger penetration. However, a wide range of physical parameters have been used in the literature. For example, the compression crosshead speeds vary from 10 to 300 mm/min and % plunger penetrations into the tofu samples from 25 to 99%. Since the rheological response depends upon the different physical parameters used. As a result, the data reported from various laboratories cannot be meaningfully compared. Beyond this problem, there is no information to indicate which single set of the physical conditions (including both sample preparations and machine operational parameters) is the most suitable to generate texture profiles of tofu that correlate the best with the sensory scores. Although correlations between the mechanical textural properties of various types of foods to that rated by the sensory panelists (*8*, *13*), the tofu products have not been studied. Since there are many types of tofu products in the market, a valid

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

correlation between mechanical testing methods and sensory evaluation would expand the usefulness of the TPA method in quality control program or in research and development. In research, comprehensive, classical rheological studies of texture characteristics using multiple factorial designs are the best. However, they are very time-consuming since such studies would involve controlling several physical factors and using different mechanical principles or methodologies (*12*), and therefore not suitable for quality control. If a single set of mechanical conditions could produce good sensory-correlated textural data, the time saved in quality control will be very significant. Thus, investigating and defining suitable Instron machine operation conditions that produce numerical data that correlates well with sensory characteristics is very important for the tofu industry.

Our objective was to investigate how selected Instron mechanical parameters and specimen preparations (middle no-skin part or the part with skin) affected textural properties of tofu products, and how well the mechanical hardness and springiness correlated with that measured with a descriptive sensory method.

Materials and Methods

Materials

We used thirteen commercial tofu products, ranging from silken, soft, regular, firm, and extra-firm products (Table I).

Mechanical Method

Two levels of plunger penetration (50% and 75% deformation) and four compression speeds (20, 60, 100, 200 mm/min) were used to determine the force-time response using the TPA method with an Instron universal testing machine (Model 1011, Instron Co., Canton, MA). A round disc plunger with 10 cm in diameter and a smooth surface (Figure 2) was used. Refrigerated tofu products were cut with a stainless cylindrical cutter with 44 mm in diameter and 15 mm in height (Figure 3). The temperature of tofu was 10-15 C during the course of compression analysis. The plunger moved down to deform the tofu to the position of 0.75 cm and 0.375 cm from the bottom of the original tofu gel for the 50 and 75% penetrations, respectively. In each measurement, two compressions were carried out simulating two bite (chew) cycles. During the compressions, the entire tofu specimen was under the disc plunger. Two different tofu specimen preparations are analyzed, one containing the outer skin layer at the bottom side and the other with the outer skin cut off (without the outer skin layer). Each parameter condition was carried out in triplicate. The Bourne's textural profiles, including hardness, fracturability, springiness, cohesiveness, and gumminess, were calculated (*10*).

Table I. Properties of Tofu Products Studied

Tofu Products	*Packaging Method*	*Coagulant Type (in order of decreasing concentration)*	*Skin or Not*	*Protein Content (%)*
Frieda brand				
Soft	Packed in water	$CaSO_4$ and GDL	No skin	5.9
Firm	Packed in water	$CaSO_4$	With Skin	7.1
Extra Firm	Packed in water	$CaSO_4$ and $CaCl_2$	With skin	11.8
Hinoichi brand				
Soft	Packed in water	$CaSO_4$ and GDL	No skin	5.9
Regular (Medium)	Packed in water	$CaSO_4$	With skin	8.2
Firm	Packed in water	$CaSO_4$	With skin	9.4
Extra Firm	Packed in water	$CaSO_4$ and $CaCl_2$	With skin	9.4
Mori-nu brand				
Soft	Formed in the box	GDL, $CaCl_2$	No skin	4.8
Firm	Formed in the box	GDL, $CaCl_2$	No skin	7.1
Extra Firm	Formed in the box	GDL, $CaCl_2$	No skin	7.5
Vitasoy (Shanshui) brand				
Silken (Soft)	Filled tofu, curd was formed in the box	GDL	No skin	4.4
Regular	Packed in water	$CaSO_4$ and $MgCl_2$	With skin	8.2
WhiteWave brand				
Organic Soft	Packed in water	$MgCl_2$, $CaSO_4$	With skin	12.1

(Reference (*6*))

Sensory Method

Details of the method were described in Yuan and Chang (*6*). A continuous linear-scale descriptive flavor intensity method was used to characterize sensory hardness and springiness (*14*). Seven panelists who were familiar with tofu products were trained using known commercial references with different firmness qualities to assist in defining the hardness and springiness scales. Panelists were trained in three sensory sessions at in two weeks, and each sample tofu testing was conducted twice at different dates. Three-digit random numbers were used to code the samples. Sensory panelists were advised to taste only the center portion to avoid the skin effect of the pressed tofu products. The soft types had a

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

semi-solid characteristic. The firm and regular types of tofu were intermediate in between the semi-solid and solid foods, whereas the extra-firm tofu approached the solid food characteristics. In the evaluation of the firm and extra-firm tofu, the panelists were trained to judge the sensation of hardness as the force (energy) to bite the foods with the molar teeth, whereas for the soft semi-solid tofu could also be sensed by measuring the force in compressing the tofu against the palate with the tongue (*12*). The springy (elastic) sensation was measured by the degree to which the original forms were returned after a slight compression in the mouth (*15*). All panelists except one were frequent consumers of tofu and they had no problem in determining these two texture properties.

Statistical Analysis

Data was analyzed by ANOVA (*16*). Duncan multiple range test was used to analyze effect of treatments at $p \leq 0.05$. Pearson's correlation coefficient was used to determine the correlationships between Instron mechanical and sensory characteristics of tofu hardness and springiness values. Euclidean analysis was used to determine the trend pattern closeness between the sensory and Instron mechanical hardness values.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

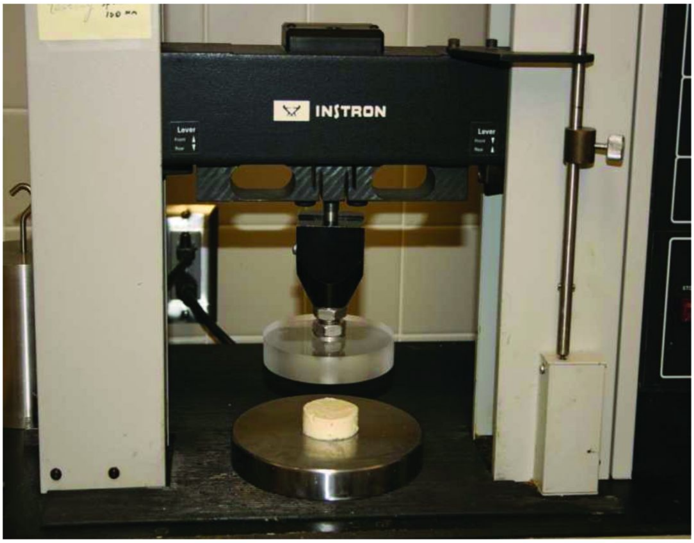


Figure 2. Instron Universal Testing machine showing the plastic plunger pressing down on a piece of tofu.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

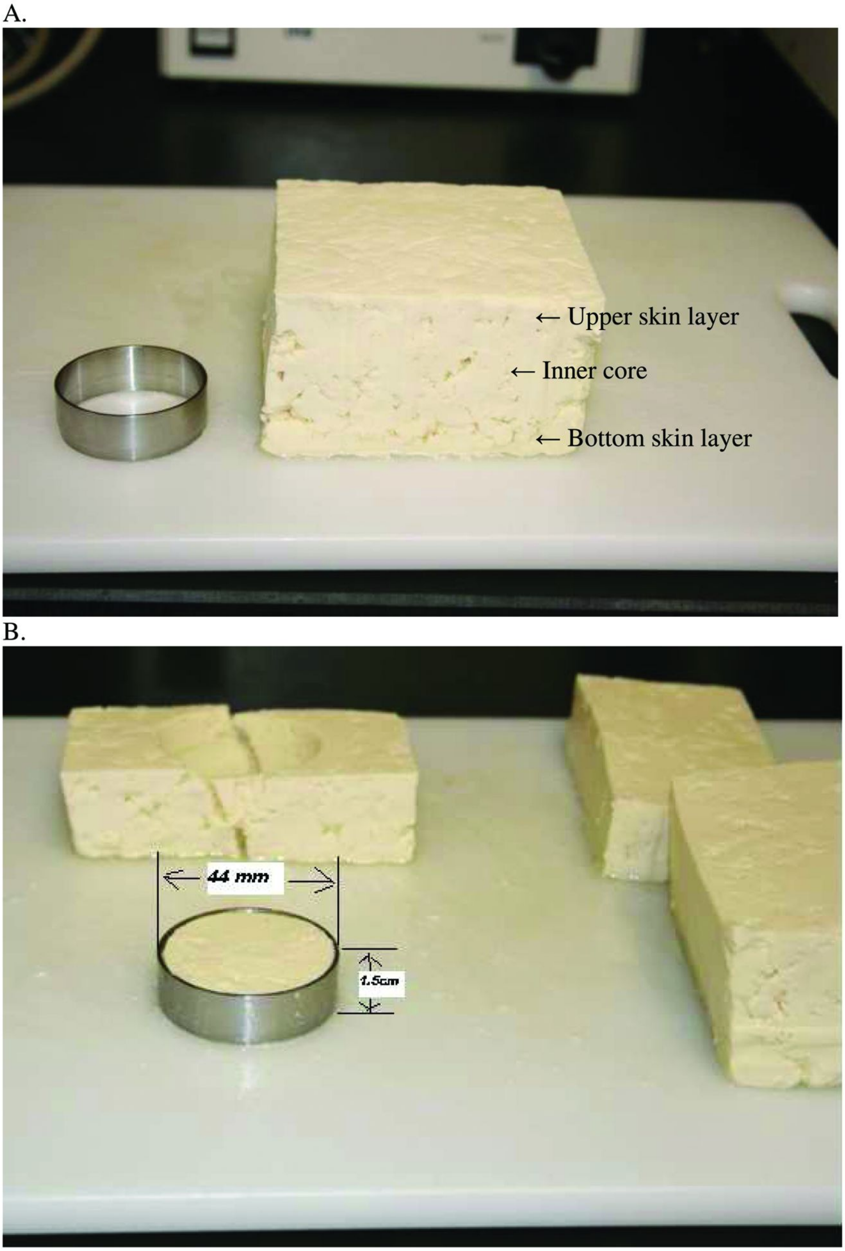

A.
← Upper skin layer
← Inner core
← Bottom skin layer
B.
44 mm
1.5cm

Figure 3. A. Shows tofu and layers of tofu and the stainless cutter. 3.B. shows tofu cut by the cutter and its dimension (44 mm x 15 mm).

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Results and Discussion

Textural profile analysis (TPA) using Instron universal testing machine has been considered as a standard method for characterizing the textural properties of a wide variety of foods (*8*, *12*, *13*). Although TPA had not been correlated with human sensory data for tofu products, most researchers characterized tofu texture using Instron TPA methods. Since we selected two plunger penetrations and four crosshead speeds and two specimen preparation methods for measuring 13 types of commercial tofu products with 3-4 determinations for each condition, therefore, many combination conditions were tested for the analysis of tofu texture profiles.

Commercial tofu products vary in their protein contents, coagulant types and packaging methods (Table I). Figure 4 shows a typical Instron mechanical force-time curve consisted of two bites. Only one peak showed if the sample did not have a clear fracture point in the first bite cycle. However, two peaks showed when tofu had a clear fracture point. In the second bite cycle a single peak was formed in all types of tofu since the tofu mass had been broken in the first bite. Soft tofu normally had a sharp fracture peak and the regular tofu had a noticeable fracture bump. The pressed type firm or extra-firm tofu did not have a fracture point. However, the GDL coagulated firm and extra-firm tofu products, which were manufactured in the Tetrapak® boxes (no pressing) also showed clear fracture peaks.

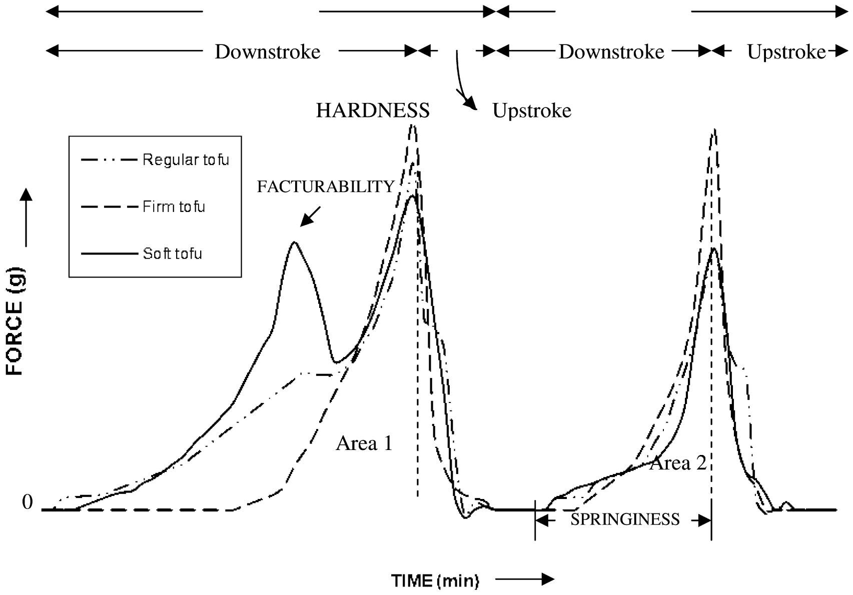


Figure 4. Typical Instron force-time curve of three major types of tofu products when plunger penetration was 75%. (Reference (6))

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Effect of Crosshead Speed

Under 75% plunger penetration, the hardness of all no-skin tofu products (including seven pressed tofu products with the skin cut off during specimen preparation) was affected by the cross-head speed (Table II). The average firmness (under all speeds) of silken and soft tofu types ranged from approximately 1.6 to 4.6 Kg force, except the WhiteWave's Organic Soft Tofu, which had a firmness of about 22 Kg force, which was even higher than that of other company's firm and extra-firm tofu products. Therefore, aberrantly high value of the WhiteWave soft tofu was excluded in sensory and correlation analysis. The average firmness of regular tofu ranged from about 7-10 kg force, while the firm tofu ranged from 4-7 kg force. On the other hand, the firmness values of the extra firm type ranged from 6 to 14 kg force. Therefore, the naming of the tofu firmness depended upon companies since there were significant firmness crossovers in all three categories of regular, firm and extra-firm tofu products.

Table II. Effect of Crosshead Speed with 75% Plunger Penetration on Tofu Hardness (kg force)*

Tofu Products	*Crosshead Speed (mm/min)*				
	20	*60*	*100*	*200*	*Average*
Shanshui Silken	1.37^{Ha}	1.64^{Ia}	1.82^{Ia}	1.87^{Fa}	1.68
Shanshui Regular	10.13^{Dab}	11.5^{Da}	8.42^{Db}	11.67^{Ca}	10.33
Hinoichi Soft	2.87^{GHc}	3.62^{Hb}	3.66^{Hb}	3.88^{Ea}	3.39
Hinoichi Regular	7.13^{Ea}	7.63^{Ea}	7.5^{Ea}	7.23^{Da}	7.37
Hinoichi Firm	5.13^{Fa}	6.87^{EFab}	7.13^{EFab}	8.4^{Da}	6.72
Hinoichi Extra Firm	15.83^{Ba}	16.0^{Ba}	15.67^{Ba}	17.38^{Ba}	16.17
Mori-Nu Soft	1.33^{Hb}	1.65^{Ia}	1.68^{Ia}	1.77^{Ea}	1.61
Mori-Nu Firm	3.75^{FGb}	4.50^{Ga}	4.62^{Ga}	4.73^{Ea}	4.40
Mori-Nu Extra Firm	5.31^{Fc}	6.17^{Fb}	6.53^{Fa}	6.83^{Da}	6.21
Frieda's Soft	4.80^{Fa}	4.60^{Ga}	4.60^{Ga}	4.61^{Ea}	4.65
Frieda's Firm	5.2^{Fc}	5.2^{Gc}	6.77^{EFb}	8.2^{Da}	6.74
Frieda's Extra Firm	13^{Cb}	14.5^{Cb}	14.67^{Cb}	16.67^{Ba}	14.79
Organic Soft	19.67^{Ab}	20.67^{Ab}	19.92^{Ab}	28.67^{Aa}	22.23

* The pressed tofu was cut to remove the skin layers before Instron measuring. Means followed by different capital letters A, B, C… in the same column indicate significant differences among different types of tofu in terms of speed 20, 60, 100 and 200 at $p \leq 0.05$. Means followed by different lower case letters a, b, c… in the same row indicate significant differences among different speeds for each type of tofu at $p \leq 0.05$. Source: Reference (*6*)

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Table III. Instron Textural Profiles of Tofu Products Measured with 75% Plunger Penetration and Crosshead Speed of 100 mm/min[*]

Type of Tofu	*Hardness, Kg force*	*Springiness, cm*	*Fracturability, Kg force*	*Cohesiveness, dimensionless*	*Gumminess, Kg force*
Table 3.a. Tofu Specimens With Skin Cut Off					
Shanshui Silken	1.86^{I}	0.31^{E}	1.82^{D}	0.08^{EF}	0.15^{EF}
Shanshui Regular	8.40^{D}	0.66^{D}	N/A[**]	0.20^{D}	1.68^{C}
Hinoichi Soft	3.64^{H}	0.32^{E}	2.53^{C}	0.11^{E}	0.40^{EF}
Hinoichi Regular	7.50^{E}	0.55^{B}	N/A[**]	0.25^{C}	1.85^{C}
Hinoichi Firm	6.95^{EF}	0.53^{BC}	N/A[**]	0.24^{C}	1.73^{C}
Hinoichi Extra Firm	15.67^{B}	0.84^{A}	N/A[**]	0.38^{A}	5.99^{B}
Mori-Nu Soft	1.68^{I}	0.29^{E}	1.68^{D}	0.07^{F}	0.12^{E}
Mori-Nu Firm	4.62^{G}	0.47^{CD}	4.62^{B}	0.08^{EF}	0.37^{EF}
Mori-Nu Extra Firm	6.53^{F}	0.47^{CD}	6.53^{A}	0.07^{F}	0.46^{E}
Frieda's Soft	4.60^{G}	0.44^{D}	2.50^{C}	0.19^{D}	0.90^{D}
Frieda's Firm	6.77^{EF}	0.51^{BCD}	N/A[**]	0.25^{C}	1.69^{C}
Frieda's Extra Firm	14.67^{C}	0.83^{A}	N/A[**]	0.40^{A}	5.87^{B}
Organic Soft	19.92^{A}	0.80^{A}	N/A[**]	0.33^{B}	6.50^{A}
Table 3.b. Tofu Specimens With Skin					
Shanshui Regular	12.13^{C}	0.84^{AB}	NA[**]	0.35^{BC}	4.29^{CD}
Hinoichi Regular	11.80^{C}	0.49^{C}	NA[**]	0.24^{D}	2.84^{D}
Hinoichi Firm	12.33^{C}	0.72^{B}	NA[**]	0.36^{BC}	4.50^{C}
Hinoichi Extra Firm	19.25^{B}	0.85^{AB}	NA[**]	0.43^{AB}	8.35^{B}
Frieda's Firm	10.50^{C}	0.41^{C}	NA[**]	0.29^{CD}	3.06^{CD}
Frieda's Extra Firm	20.33^{B}	0.95^{A}	NA[**]	0.51^{A}	10.30^{A}
Organic Soft	22.67^{A}	0.89^{A}	NA[**]	0.42^{B}	9.52^{AB}

[*] Data are means of four determinations. No-skin specimens were cut from the middle portion of the tofu cake. Means followed by different capital letters A, B, C… indicate significant differences among different types of tofu in terms of hardness, springiness, cohesiveness, gumminess and fracturability at $p \leq 0.05$. [**] Specimens had no significant breaks during the course of compression. Therefore, fracturability was not available. Source: Reference (*6*)

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

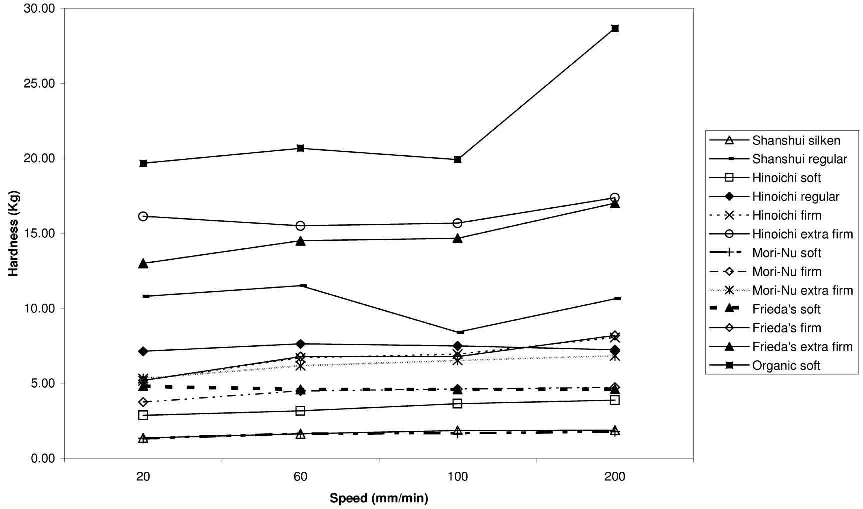


Figure 5. Effect of crosshead speed on the hardness measured at 75% penetration of thirteen commercial no-skin tofu specimens (both top and bottom skin layers were cut off). **(***Reference (6))*

Generally, the Instron mechanical hardness values increased with the increase in the firmness labels of the products in each brand. A positive correlation (r = 0.87, p <0.01) existed between protein contents of the tofu and the average hardness values for all products. The compression speeds affected differently the Instron's response in hardness values for different tofu products, with increased hardness values at higher speeds (Table III**,** Figure 5). This was true for four products from 20 to 60 mm/min, and four products from 100 and 200 mm/min. Only two products increased and one product decreased in hardness values when the speed increased from 60 to 100 mm/min. The average hardness values of all products were the most similar using crosshead speeds between 60 and 100 mm/min.

As compared to the effect of crosshead speed on hardness, the effect pattern of speed on springiness was more irregular with increasing or decreasing trends when the speed was increased. The effect of speed on cohesiveness (Figure 6) showed that cohesiveness decreased or not changed when speed was increased. This was understandable since the force impact in the first bite was greater to break up the tofu body when the penetration speed was increased. The decreases in cohesiveness with speed were higher for the regular, firm and extra-firm tofu products, which were manufactured with a pressing step, during which tofu curd had been partially broken. It is interesting to note that when a high speed as 200 mm/min was used, the differences in cohesiveness among tofu products became smaller and not significant at ($p < 0.05$). This means that cohesiveness of tofu if measured at 200 mm would have much less differences among tofu products than that measured at lower speeds. Therefore, 200 mm/min was a less desirable condition for TPA analysis of tofu cohesiveness. Ideally, mechanical parameters chosen should be sensitive to reflect the texture differences among tofu products.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

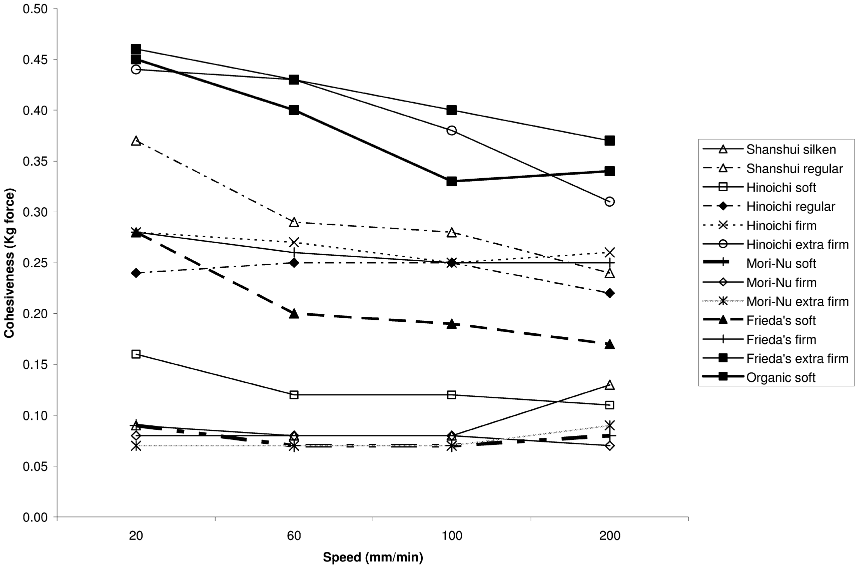


Figure 6. Effect of crosshead speed on the cohesiveness measured at 75% penetration of thirteen commercial no-skin tofu specimens (both top and bottom skin layers were cut off). (Reference (6))

The effect of speed on the fracturability values were tested on soft and silken tofu and the Morinu tofu, which were manufactured without curd breaking and pressing. Increasing trends were observed as the speed was increased (*6*). The gumminess responses as affected by the speed were irregular with some tofu products decreased while others increased in gumminess. This was understandable since gumminess was related to hardness and cohesiveness, which was irregular for the tofu types tested.

Based on the above studies, it can be concluded that the speeds 20 to 100 mm/min were more suitable for measuring tofu texture profiles than 200 mm/min. Considering the time saving factor in the operation of the Instron machine, the speed of 100 mm was the most suitable for tofu profile analysis.

Effect of Specimen Preparation: With Skin versus No-Skin Tofu

Tofu products with a skin layer were the types that were made by a combination step of curd breaking and hard-pressing during tofu manufacturing. Table III(a) reports the textural profiles of the tofu without skin measured with 75% plunger penetration and crosshead speed of 100 mm/min and Table III(b) reports the profiles of the tofu with skin at the bottom as determined using the same % penetration and cross-head speed (*6*). These data could serve as the references for future research or for quality control comparison of these selected types of tofu products. Comparing the results of tofu with skin to that with skin removed from the same types of tofu, the tofu with skin had higher hardness, springiness, cohesiveness, and gumminess values. However, the tofu with skin

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

did not show the fracturability peak, indicating the skin can protect tofu from be fractured by physical impact.

The detailed results of the specimen preparations on the texture profiles are not shown here. In general, skin-tofu products had higher texture values than the no-skin tofu. On an average of the seven tofu types that had the skin in their original form, when determined at 75% penetration with 100 mm/min speed, the hardness, cohesiveness, springiness, and gumminess of the tofu products with skin had approximately 36, 27, 6 and 66% higher values than that of the same products with the skin cut off. The springiness had the lowest differences (6%) between the tofu products with skin and with the skin removed.

Effect of the Percentage of Plunger Penetration (Degree of Deformation)

The detailed results for the effect of speed with 75% versus 50% penetration also are not reported here. Comparing to 75% penetration, the 50% penetration force response was decreased in hardness and gumminess but increased in springiness and cohesiveness (*6*). Based on the average values of all tofu products studied at 100 mm/min penetration speed, hardness and gumminess values were decreased by 58 and 33%, respectively, and cohesiveness and springiness were increased by 96 and 29%, respectively, when penetration was decreased from 75 to 50%. The changes in the above four texture variables were reasonable since decreasing deformation to 50% decreased force impact so that most of the gel body texture was maintained after the first-bite cycle, leading to higher cohesiveness and springiness than that determined by 75% penetration. Tofu has a three-dimensional network, in which water is held. When the structure of the network was damaged by the force impact, some water was released from the matrix to reduce the ability to return to the original gel shape. More damage caused more water leakages (*12*). Since the deformation by 50% plunger penetration caused less destruction on the body matrix than that did by 75%, the cohesiveness and springiness measured at 50% penetration would naturally be higher than that measured at 75%. All tofu products did not show fracture peaks at the 50% penetration condition, indicating that this level of compression was not deep enough to cause fracture during the course of the plunger movement. Although the mean data of the 50% penetration experiments are not presented here, the data were used in the statistical analyses for correlations among the physical variables and sensory scores.

Sensory Evaluation

Sensory scores of the hardness of tofu varied widely among the selected types of tofu products. The results in Table IV showed sensory hardness and springiness scores of all tofu products, excluding the WhiteWave's Organic Soft product, which was an outlier in the firmness claim of the soft tofu products. Just as that was exhibited in the Instron mechanical analyses, there were significant differences ($p \leq 0.05$) in sensory hardness (intensity scores ranged from 1.25 to 3.35) and springiness (intensity scores ranged from 1.25 to 3.2) among the soft or silken tofu products made from different companies. Among the three extra-firm tofu products, the two products made by pressing had significantly higher sensory hardness and springiness than that of the Mori-Nu Extra-Firm, which was coagulated and formed in the box without going through a pressing step. The sensory hardness and springiness scores of the extra-firm products were parallel with their protein contents. However, there was an increasing hardness trend with increases in the firmness classes labeled within each company's products. Table IV shows that sensory scores for springiness, in some cases, were parallel to the hardness scores. This is logical since tofu springiness values and the hardness values of the Instron mechanical analyses were highly correlated.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Table IV. Descriptive Sensory Scores for the Hardness and Springiness Characteristics of Twelve Commercial Tofu Products

Type of Tofu	*Hardness*	*Springiness*
Shanshui Silken	1.25E	1.25F
Shanshui Regular	7.9B	7.8B
Hinoichi Soft	2.3E	2.3EF
Hinoichi Regular	5.85C	6.1C
Hinoichi Firm	6C	6.15C
Hinoichi Extra Firm	9.4A	9.6A
Mori-Nu Soft	1.25E	1.4F
Mori-Nu Firm	4.1D	3.75D
Mori-Nu Extra Firm	6.1C	5.2C
Frieda's Soft	3.35D	3.2DE
Frieda's Firm	5.75C	5.75C
Frieda's Extra Firm	9.3A	9.1A

* Each run of the sensory evaluations was the average of the scores of seven panelists. The means were calculated from two replicates of sensory evaluation. * Means followed by different capital letters A, B, C… indicate significant differences among 12 different types of tofu in terms of hardness and springiness at $p \leq 0.05$. Source: Reference (*6*)

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Table V. Correlation Coefficients of Springiness and Hardness between Instron Values and Sensory Scores

Hardness	*Springiness*	*Penetration (%)*	*Samples with or without skin*[1]	*Speed*	*Tofu types*
0.94*	0.89*	75	With skin	20	12
0.95*	0.91*	75	With skin	60	12
0.95*	0.91*	75	With skin	100	12
0.94*	0.93*	75	With skin	200	12
0.93*	0.92*	75	No skin	20	12
0.96*	0.98*	75	No skin	60	12
0.94*	0.98*	75	No skin	100	12
0.95*	0.97*	75	No skin	200	12
0.93*	-0.30	50	With skin	20	12
0.89*	0.33	50	With skin	60	12
0.86*	0.56	50	With skin	100	12
0.91*	0.75	50	With skin	200	12

* Significant at $p \leq 0.05$. [1] Specimens were prepared by two methods. The 'with skin' group denotes the samples were cut with the top skin layer removed but the bottom skin layer remained intact. The 'no skin' group denotes that the specimens were cut from the middle portion of the tofu without containing the top or bottom skin layers. Source: Reference (*6*)

Correlations between Mechanical and Sensory Data

The degree of plunger penetration had a significant impact on the correlations of hardness and springiness between Instron mechanical values and sensory scores (Table V). Hardness values obtained at 75% of plunger penetration correlated significantly with the sensory hardness scores (r=0.93 to 0.96 for all four speeds with or without skin, $p \leq 0.05$). Hardness obtained by 50% penetration at all speeds also had significant correlations with the sensory hardness scores. However, springiness obtained by the 50% penetration method did not correlate significantly with the sensory data, whereas 75% penetration had significant correlations, with highest coefficient of 0.98 when Instron conditions of 60-100 mm/min crosshead speeds, 75% penetration, and no-skin tofu were used. Mechanical hardness values obtained using tofu specimens without skin, 75% penetration and 60 mm/min speed had the highest coefficient (r = 0.96) with the sensory hardness scores. And the highest correlation coefficient between mechanical springiness values and sensory springiness scores was 0.98 for 75% penetration with 60 mm and 100 mm/min speeds, respectively. Szczesniak and others (*8*) reported a curvilinear relationship between the sensory hardness scores of nine types of foods (ranging from the soft scale of cream cheese, hard cooked egg white, and frankfurter to high hardness scales of fresh carrot, brittle peanut

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

and candy) with their mechanical hardness values. Our study showed that the relationship between the mechanical and the sensory hardness was approximately linear. This study is the first in literature reporting this linear phenomenon for the 12 selected types of tofu products.

Euclidean distance analysis is a common method used for analyzing the closeness of the cluster patterns of multiple variables (*17*). Results showed the squared Euclidean distance between the Instron hardness and the sensory scores for speed 20, 60, 100 and 200 mm/min was 139, 184, 130 and 252, respectively (*6*). The smaller the summation values between two lines indicate closer similarities in the two sets of the texture analytical patterns (Figure not shown). Therefore, among the four speeds, 100 mm/min produced the least, and 200 mm/min the largest summation of the squared Euclidean distance between each pair of the Instron and sensory values of the 12 tofu products. Therefore, among the four speeds, 100 mm/min produced the closest pattern to the sensory pattern of the 12 tofu products. Hardness is the most important tofu textural characteristic, on which market classes of tofu are based. Tofu products do not have officially approved standards of identity. Our findings provide a sound basis towards the standardization of textural properties for marketing tofu products or for quality control purpose.

Conclusion

This study provided a theoretical basis for the selection of the physical/mechanical parameters for determining texture properties by Instron universal testing machine. Using consistent physical parameters is important, particularly for interlaboratory data comparisons. Based on variance, correlations between mechanical and sensory scores, and the time-saving factor, we recommend using the combination conditions of 75% penetration, crosshead speeds of 100 mm/min and no-skin specimen for Instron texture profile analysis of tofu. In addition to the significance in the standardization of mechanical parameters, the described texture analysis methods when used with the small-scale tofu making method provide a valid method for the evaluation of suitability of soybean materials for tofu making.

Acknowledgments

ND AES Hatch Project 1908, North Dakota Soybean Council and USDA CSREES-NRI (NIFA-AFRI) CGP No. 2001-10853 provided funding for this project. David Lo, Jia Guo and Zhisheng Liu provided technical help for this work.

References

1. Chang, S. K. C. Soymilk and Tofu Manufacturing. In *Handbook of Food Product Manufacturing*; Hui, Y. H., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2007; Chapter 95, pp 1063−1089.

2. Food Labeling: Health Claim; Soy Protein and Coronary Heart Disease. FDA, 21 CFR Pt. 101. 1999. Final Rule. *Fed. Regist.* **1999**, *64* (206), 57700−57733.
3. Wang, R. C. C.; Chang, K. C. Physicochemical properties and tofu quality of soybean cultivar Proto. *J. Agric. Food Chem.* **1995**, *43*, 3029–3024.
4. Murphy, P. A.; Chem, H. P.; Hauck, C. C.; Wilson, L. A. Soybean protein composition and tofu quality. *Food Technol.* **1997**, *51* (3), 86–88, 110.
5. Cai, T. D.; Chang, K. C.; Hou, H. J.; Ji, M. P. Comparison of bench and production scale methods for making soymilk and tofu from 13 soybean varieties. *Food Res. Int.* **1997**, *30*, 659–668.
6. Yuan, S. H.; Chang, K. C. Texture profile of tofu as affected by Instron parameters and sample preparation, and correlations of Instron hardness and springiness with sensory scores. *J. Food Sci.* **2007**, *72*, S136–145.
7. Friedman, H. H.; Whitney, J. E.; Szczesniak, A. S The texturometer-a new instrument for objective texture measurement. *J. Food Sci.* **1963**, *28*, 390–396.
8. Szczesniak, A. S.; Brandt, M. A.; Friedman, H. H. Development of standard rating scales for mechanical parameters of texture and correction between the objective and the sensory methods of texture evaluation. *J .Food Sci.* **1963**, *28*, 397–403.
9. Bourne, M. C. Texture profile analysis. *Food Technol.* **1978**, *32* (7), 62–66.
10. Bourne, M. C. *Food Textures and Viscosity: Concept and Measurement*; Academic Press: New York, 1982.
11. Szczesniak, A. S.; Bourne, M. C. Letters. *J. Food Sci.* **1995**, *60* (6), vii.
12. Lee, Y. C.; Rosenau, J. R.; Peleg, M. Research note rheological characterization of tofu. *J. Texture Stud.* **1983**, *14*, 143–154.
13. Henry, W. F.; Katz, M. H.; Pilgrim, F. J.; May, A. T. Texture of semi-solid foods: sensory and physical correlates. *J. Food Sci.* **1971**, *36*, 155–161.
14. Stone, H.; Sidel, J. L. *Sensory Evaluation Practices*, 2nd ed.; Academic Press, Inc.: San Diego, CA, 1993.
15. Cardello, A. V.; Maller, O. Sensory Texture Analysis: An Integrated Approach to Food Engineering. In *Food Texture*; Moskowitz, H. R., Ed.; Marcel Dekker, Inc.: New York, 1987; pp 177−216.
16. *SAS User Guide: Statistics*; SAS Institute, Inc.: Cary, NC, 2005.
17. Macias, F. A.; Marin, D.; Oliveros-Bastidas, A.; Castellano, D.; Simonet, A. M.; Molinllo, J. M. G. Structure-activity relationships (SAR) studies of benzoxazinones, their degradation products and analogues. Phytotoxicity on standard target species (STS). *J. Agric. Food Chem.* **2005**, *53*, 538–548.

Downloaded by CORNELL UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch015

Downloaded by OHIO STATE UNIV LIBRARIES on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch016

Chapter 16

Soybean Phytate Content and Its Influence on Tofu Texture

Takahiro Ishiguro*,[1] and Tomotada Ono[2]

[1]Food Research Center, Asahimatsu Foods Co. Ltd., 1008 Dashina, Iida, Nagano, Japan
[2]Faculty of Agriculture, Iwate University, Ueda 3, Morioka, Iwate, Japan
***Ishiguro@asahimatsu.co.jp**

The variation factor in soybean phytate content and its influence on tofu texture was investigated. Twenty-seven soybean varieties were grown in a drained paddy field and upland field, and then their protein and phytate contents were determined using the FT-IR method. 12 soybean varieties grown in the drained paddy field contained significantly more phytate than those in the upland field. Variation in the phytate contents due to the different varieties was also observed. Therefore phytate content was affected by both environmental and hereditary factors. The increase of phytate concentration in soymilk shifted optimal coagulant concentration in both Mg and GDL tofu. The data showed the variation of phytate content influenced on tofu texture.

Introduction

Tofu has been a popular food in some Asian countries since ancient times. Its consumption is now increasing as it is recognized as a wholesome food in all over the world. The quality of tofu is mainly evaluated on the basis of its texture properties. Phytate is considered to affect the tofu texture by reacting with protein and coagulants such as the magnesium or calcium salts (*1*) . Phytate occurs in many grains and legumes. Binding of phytate to soy protein has been reported to influence the physicochemical properties of the protein. Katoh *et.al.* (*2*) reported that the removal of phytate from soy protein increased the surface hydrophobicity and emulsifiability of the protein. Those phenomena suggest that the linkage of

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by OHIO STATE UNIV LIBRARIES on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch016

phytate to protein could change the existing form of protein in soymilk and then affect tofu texture.

A few studies have discussed the influence of phytate on tofu texture. Hou and Chang (*3*) reported that phytate contents in soymilk have little effect on pressed tofu. Saio *et.al.* (*4*) reported that increasing the phytate contents in soymilk caused a decrease in hardness and an increase in yield of tofu. Liu and Chang (*5*) have reported that phytate contents in soymilk have a correlation with optimal coagulant concentration.

Soybean cultivation is increasing in fields that have been converted from paddy fields for rice (called "drained paddy field" in this study) in Japan. However, drained paddy fields may be different from the primary soybean field (called "upland field" in this study) based on some conditions such as the soil properties. For example, it is known that soil in the drained paddy field have higher phosphate adsorption coefficient than in the upland field. The difference in the cultivation environments may affect several characteristics of the soybean. Therefore, it will then affect the tofu quality.

In this study, in order to investigate the effect of soybean variety and growing condition on phytate contents, we cultivated 27 varieties of soybean in both a drained paddy field and upland field, and then their phytate contents were determined. Then we further investigated the influence of the soybean phytate content on the tofu texture.

Materials and Methods

Materials

Twenty-seven Japanese varieties of soybeans (Figure 1) were grown in a drained paddy field and upland field. These soybeans were used to investigate the changes of the phytate contents.

A variety of Suzuyutaka was used for examining the influence of the phytate content on the tofu texture.

Determination of Phytate in Soybean

The phytate content was measured by the method of Ishiguro *et al.* (*6*). Each soybean (about 10 g) was soaked in deionized water for 18 h at 4°C. The swollen bean was ground (11,400 rpm, 4 min) into a homogenate with 8 times the water vs. the soybean dry weight using an Oster blender (Oster Co, Milwaukee, USA), and the homogenate was then filtered through a defatted cotton sheet. The filtrate was designated as the raw soymilk. Phytate in the raw soymilk was completely precipitated by the addition of calcium under alkaline conditions (pH 11.5). The precipitate was dissolved in citrate buffer (pH 6.0) and then the IR spectra were measured with the ATR accessory. The phytate content was determined by the absorbance at 1070cm^{-1}.

Downloaded by OHIO STATE UNIV LIBRARIES on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch016

Preparation of Tofu

The soybeans were soaked in deionized water for 18 h at 4°C. Each swollen bean was ground (11,400 rpm, 4 min) into a homogenate with 6 times the water vs. the soybean weight using the Oster blender (Oster Co, Milwaukee, WI), then an antifoam-emulsion (Antifoam-AF-Emulsion, Nakarai Chemicals Ltd.) was added (about 80 mg for 200ml of soymilk) during the grinding. The homogenate was filtered through a defatted cotton sheet. The filtrate is the raw soymilk. In order to prepare the soymilks having different phytate contents, the potassium phytate solution (pH 7.0) was added to the raw soymilk. Those soymilks have same composition of protein, calcium, magnesium, and other contents expect phytate. Soymilk was prepared by heating the raw soymilks in a boiling bath for 5 min above 95 °C, and then quickly cooled to room temperature with iced water.

The Soymilk (25 mL) was degassed by vacuum-aspiration and cooled to 4 °C. Each 1.0 mL of various concentrations of $MgCl_2$ or Glucono-1-5-lactone (GDL) solutions was mixed into the soymilk and the mixture was placed in a water bath (Isotemp Fisher general purpose water bath, Fisher Scientific, Boston, MA, USA) at 90 °C for 1 h.

Measurement of Tofu Texture

The tofu was placed in a refrigerator (4°C) for over 18 hours. When measuring the curd texture, the temperature of the tofu curd was at room temperature (23°C). The tofu, 13 mm height and 20 mm diameter, placed on a measuring plate. A compression test was carried out at the compression rate of 2 cm / min using a RHEOTECH FUDOH RHEOMETER (Rheotech Co., Tokyo, Japan) with a 25 mm diameter cylindrical plunger.

Statistical Analyses

Significant differences between group means were analyzed by a t-test ($p >$ 0:05) using the WinSTAT program.

Results and Discussion

Effect of Variety and Field Difference on Soybean Phytate Content

Phytate is considered to one important component in soybean that affects the tofu texture.

27 soybean varieties were cultivated in both the drained paddy field and upland field. 12 soybean varieties grown in the drained paddy field contained significantly more phytate than those in the upland field (Figure 1). The phytate contents of the other 14 verities showed no significant difference between the drained paddy and upland field. Only 1 variety of soybean grown in the upland field contained more phytate than that from the drained paddy field. The variation in the phytate contents was also observed among the different varieties. These results show that

Downloaded by OHIO STATE UNIV LIBRARIES on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch016

the phytate content is affected by both environmental and hereditary factors. This variation in phytate may have caused the scatter in the tofu texture.

The Enrei and Oosuzu varieties are known to be well suited for tofu making. On the contrary, the Hatayutaka and Tachinagaha varieties are known to be difficult for tofu making. Based on the phytate contents, Enrei and Oosuzu have no difference in the phytate contents between the drained paddy field and upland field. For Hatayutaka and Tachinagaha, the beans grown in the drained paddy field contained more phytate than those grown in the upland field. Enrei and Oosuzu have lower variation of the phytate content may cause a stable tofu making.

Effect of Soymilk Phytate Concentration on Mg Tofu Texture

Then we investigated that the influence of the variation of phytate content in soybean on tofu texture. $MgCl_2$ is a coagulant used most frequently in Japan. We measured the breaking stress of tofu made from soymilks containing various concentrations (3.3, 4.8, and 6.2 mM) of phytate with various $MgCl_2$ concentrations (5-30 mM).

Soymilk containing 3.3 mM phytate reached maximum breaking stress at 15 mM of magnesium concentration, while soymilk containing 6.2 mM phytate reached maximum breaking stress at 26 mM of magnesium concentration (Figure 2). Magnesium concentration for maximum breaking stress shifted from 15 mM to 26 mM depending upon increase of phytate content from 3.3mM to 6.2 mM (Figure 2). This coagulant concentration is recognized as optimal. It showed that fluctuation of phytate content in soybean affects Mg tofu texture.

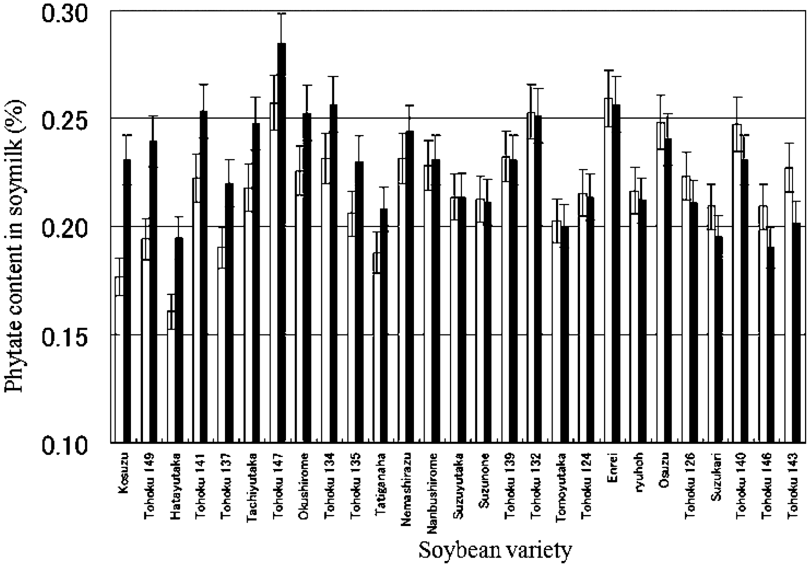


Figure 1. Phytate contents of soymilks made from soybeans grown in upland (□) and drained paddy fields (▪). Raw soymilk was prepared from each soybean to measure contents of soybean. Phytate content was measured using the method of Ishiguro et al. (5)

Downloaded by OHIO STATE UNIV LIBRARIES on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch016

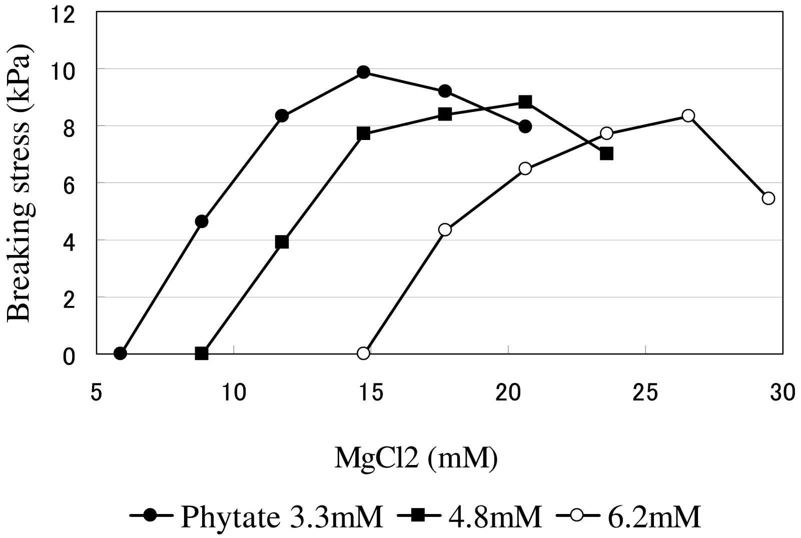


Figure 2. Breaking stress of Mg-tofu prepared from soymilks having various phytate contents

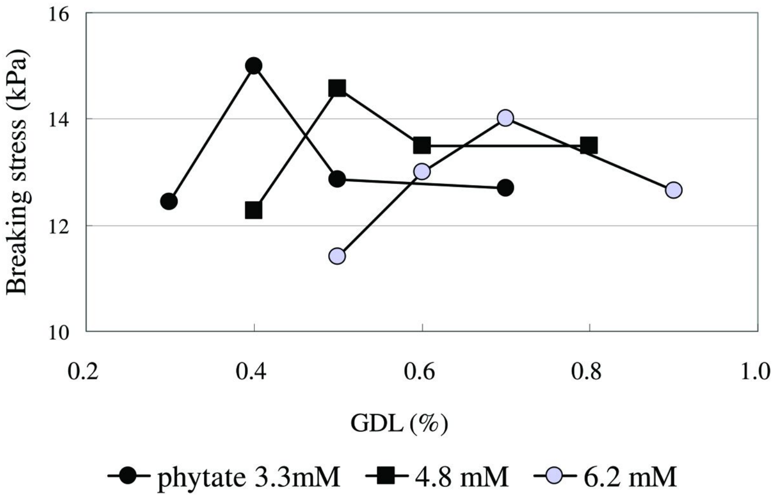


Figure 3. Breaking stress of GDL-tofu prepared from soymilks having various phytate contents

Liu and Chang (*7*) have developed the facile titration method for the examination of soymilk coagulant requirement, and they (*5*) observed that phytate content in soymilk was correlated to coagulant requirement. We confirmed this phenomenon with actual tofu and its texture.

Effect of Soymilk Phytate Concentration on GDL Tofu Texture

GDL (Glucono-1-5-lactone) is a coagulant that induces acid coagulation. It is used mainly for packed tofu. In order to examine the influence of phytate on GDL tofu texture, we measured the breaking stress of tofu made from soymilks containing various concentrations of phytate (3.3, 4.8, and 6.2 mM) with various GDL concentrations (0.3-0.9%)

Tofu containing 3.3mM phytate reached maximum breaking stress at 0.4% of GDL concentration, while tofu containing 6.2mM phytate reached maximum breaking stress at 0.7% GDL concentration (Figure 3). Since phytate have pH buffering effect, GDL requirement may be increased in high-phytate soymilk. This result showed that phytate content in soybean also affects GDL tofu texture.

Downloaded by OHIO STATE UNIV LIBRARIES on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch016

Conclusion

We concluded that phytate content in soybean was influenced by both environmental and hereditary factors. The variation of phytate content should be regarded as one of the reasons causing the variation in the tofu texture.

References

1. Toda, K.; Ono, T.; Kitamura, K.; Hajika, M.; Takahashi, K.; Nakamura, Y. Seed protein content and consistency of tofu prepared with different magnesium chloride concentrations in six Japanese soybean varieties. *Breed. Sci.* **2003**, *53*, 217–223.
2. Katoh, M.; Jin, DH.; Naganuma, T; Ogawa, T; Muramoto, K. Removal of phytic acid from soy protein isolates and its effect on protein functionality. *Daizu Tannpakushitsu Kenkyu* **2002**, *5*, 41–46, in Japanese.
3. Hou, H. J.; Chang, KC. Yield and textual properties of tofu as affected by the changes of phytate content during soybean storage. *J. Food Sci.* **2003**, *68*, 1185–1191.
4. Saio, K.; Koyama, E.; Yagasaki, S.; Watanabe, T. Protein-calcium-phytic acid relationship in soybean. Part III. Effect of phytic acid on coagulative reaction in tofu-making. *Agric. Biol. Chem.* **1969**, *33*, 36–42.
5. Liu, Z.; Chang, KC. Effect of soy milk characteristics and cooking conditions on coagulant requirements for making filled tofu. *J. Agric. Food Chem.* **2004**, *52*, 3405–3411.
6. Ishiguro, T.; Ono, T.; Nakasato, K.; Tsukamoto, C.; Shimada, S. Rapid measurement of phytate in raw soymilk by mid-infrared spectroscopy. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 752–757.
7. Liu, Z; Chang, KC. Development of a titration method for predicting optimal coagulant concentration for filled tofu. *J .Agric. Food Chem.* **2003**, *51*, 5214–5221.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Chapter 17

Soybean Components Affect Physicochemical Properties of Soymilk, Coagulation Reactivity and Tofu Texture

Effects of Glycinin Proteins, Calcium, Polysaccharides and 7S Basic Protein

Kyoko Toda,[1,*] Kyoko Chiba,[2] Kazuhiro Yagasaki,[3] Koji Takahashi,[1] Makita Hajika,[1] and Tomotada Ono[2]

[1]National Institute of Crop Science, Ibaraki, Japan
[2]Graduate School of Agriculture, Iwate University, Morioka, Japan
[3]Nagano Chushin Agricultural Experiment Station, Nagano, Japan
[*]kyokat@afrc.go.jp

When a coagulant is added to soymilk, oil globules combine with protein particles to form tofu curd. Since physicochemical properties of soymilk, such as the number and size of the protein particles, and their behavior are key factors for processing properties for tofu-making, the effect of soybean components on the physicochemical properties of soymilk was studied. Soymilk was fractionated to precipitate, soluble and floating fractions by centrifugation to study the amount of the protein particles and their interaction with lipid. The amount of precipitate increased and more triglyceride (TAG) was incorporated into the precipitate as the 11S/7S ratio increased. A similar event was observed as $MgCl_2$ concentration in soymilk increased. The stain intensity of bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that the ratio of oleosin, a membrane protein of oil body, increased in the precipitate as the $MgCl_2$ concentration or the 11S/7S ratio increased. These results indicated that protein composition affects not only the number and size of the protein particles but also their behavior. Variation of the 11S/7S ratio among different soybean cultivars affected

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

coagulation reactivity of soymilk probably due to a change of physicochemical properties of soymilk. Soymilk with more calcium, polysaccharides, and 7S basic globulin also showed more protein particulate content. Extracts of okara (a by-product of soybean processing for soymilk) mainly contain minerals, polysaccharides and 7S basic globulin. Increase in the 11S/7S ratio and addition of calcium or dialyzed extracts from okara resulted in increase in breaking stress of tofu.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

The processing properties of soybeans in tofu-making are an important concern in East Asian countries such as China, Japan, and Korea, where tofu is the main processed soybean product. The breaking stress of tofu (tofu consistency) is one of the most critical factors for the processing properties. Tofu is made from soymilk by adding coagulant such as glucono-delta lactone (GDL) and magnesium. Soymilk protein consists of particulate and soluble fractions (*1*). Guo *et al.* (*2*) reported that soymilk lipids were mainly present in the particles of raw soymilk and that they were released as oil globules when heated to above 90°C. In coagulation reaction, the incorporation of lipids into the coagulum of soymilk is triggered by the conjugation of oil globules with protein particles (*3*). It has been found that the breaking stress of tofu is dependent upon the amount of protein particles in the soymilk (*4*). Hence the number and size of the protein particles and their behavior are key factors for processing properties for tofu-making, as Ono has proposed (*5*).

Protein particles in soymilk mainly consist of glycinin and β-conglycinin (*4*), which are major storage proteins in the soybean protein components that correspond to the 11S and 7S fractions respectively (*6*). Tofu gel made from 11S protein was harder and had greater cohesiveness and elasticity than that from 7S protein (*7*), indicating that they have different physicochemical characteristics. The 11S/7S ratio has been found to affect the formation of protein particles (*8*) and to accelerate lipid incorporation (*9*). Guo and Ono (*8*) found that protein particles in soymilk and the breaking stress of tofu increased as the 11S ratio increased.

It has also been reported that the 11S/7S ratio is significantly negatively correlated with the critical point of coagulant concentration (*10*), as determined on the basis of the volume of coagulant consumed to get the swirl to disappear at a suitable stirrer speed (*11*). In our previous study, we examined the relationship between the protein content and consistency of tofu at various concentrations of $MgCl_2$ as a coagulant, and found that when the concentration of $MgCl_2$ increased, the maximum breaking stress was significantly correlated with protein content ((*12*), see Figure 1). $MgCl_2$ concentrations for the maximum breaking stress differed by variety and cultivation conditions, indicating differences in the coagulation reactivity of soymilk. Since the $MgCl_2$ concentration is not easily changed and 0.25% $MgCl_2$ is generally used in the tofu manufacturing process, coagulation reactivity is one of main factors affecting processing properties in tofu-making, especially the texture of tofu.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

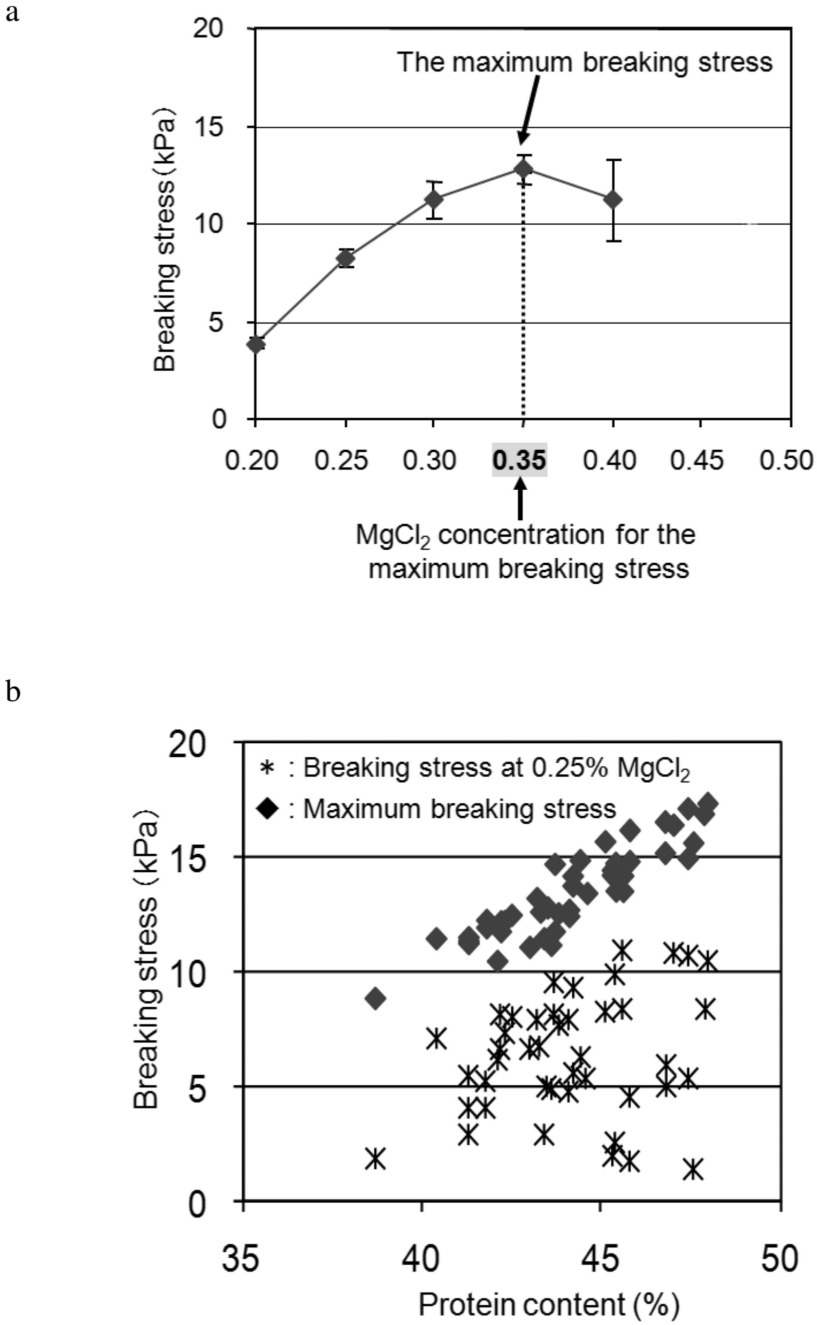


Figure 1. (a) Relationship between $MgCl_2$ concentrations and breaking stress of tofu. (b) Relationship between the protein content of soybean seeds and the maximum breaking stress of tofu (♦) and breaking stress of tofu made with 0.25% $MgCl_2$ (*).

For those reasons, in this study, we mainly focused on the difference in behavior of particles and in coagulation recativity among soybeans of different cultivars or under different cultivating conditions showing different 11S/7S ratios. We also studied the effect of other soybean components on the physichochemical properties of soymilk, using two different processing methods.

Materials and Methods

Materials

Five Japanese varieties of soybeans (*Glycine max*), Fukuyutaka, Enrei, Sachiyutaka, Hatayutaka, and Ayakogane, were obtained over several years from several locations. There were 10 samples of Fukuyutaka, 10 of Enrei, 10 of Sachiyutaka, 7 of Hatayutaka, and 10 of Ayakogane. Yumeminori, which lacks α and α′ subunits of β-conglycinin, and Tosan 205, which lacks all subunits of glycinin, were also used. Seeds were stored in plastic boxes at 5°C until used in tofu preparation, which was done within one year after harvest.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Tofu Making and Texture Profiling

Tofu was prepared as described previously (*12*). Briefly, soybeans (50 g per batch) were washed and soaked in distilled water at 20°C for 18 h. Hydrated seeds were drained and ground into homogenates with distilled water equivalent to 6 times the weight of dry seed. Raw soymilk was separated from the homogenate (okara) by centrifugation at 3,000 rpm using a centrifugal separator (SYK-3800-15A, Sanyo-Rikagaku, Tokyo, Japan) equipped with a nylon filter (120 mesh, Sanyo-Rikagaku). Raw soymilk was heated by incubation in boiling water for 6 min (for 5 min above 95 °C) and cooling in flowing water at 20°C. Soymilk made by this method is called “nama-shibori” soymilk. To examine the effects of okara on soymilk and tofu, the one portion of raw soymilk was mixed with one portion of okara and heated in a water bath for 5 min above 95 °C, followed by filtration and cooling. For convenience, the resultant soymilk was referred to as “semi-kanetsu-shibori” soymilk. The solid content of the soymilk ranged from 10% to 11%. $MgCl_2$ (hexahydrate, food grade) or GDL was added as a coagulant after the soymilk was chilled in ice-cold water. The same volume of $MgCl_2$ solution (5% of the soymilk volume) or GDL powder was added. The coagulated soymilk was incubated at 80°C for 1 h for Mg-tofu and 90°C for 1 h for GDL-tofu using a water bath and cooled in flowing water at 20°C. Cylindrical cakes of packed tofu were prepared and the tofu texture was measure according to our previous study (*12*).

Protein Analysis

A total globulin fraction was prepared following the method of Yagasaki *et al.* (*13*) with minor modification. Briefly, soluble proteins were extracted by homogenizing 2 mg of soybean powder in 0.02 M tris hydrochloric acid (Tris-HCl) (pH8.0), followed by precipitation of globulins with 15mM CH_3COONa(pH

4.5). The total globulin fraction was dissolved in 0.02 M Tris HCl (pH8.0) containing 0.32% sodium dodecyl sulfate (SDS), 7M urea and 2% (by volume) 2-mercaptoehanol. The protein was separated by SDS-PAGE (*14*) and stained with Coomassie brilliant blue G-250 (CBB G250). The stained protein bands in the gel were scanned with a scanner, and then the stained intensities were analyzed using PC software (Lane Analyzer, ATTO, Tokyo).

Evaluation of Amount and Size of Particles

The relative particulate protein content was determined by the difference in the protein concentrations of the soymilk samples before and after centrifugation at 156,000 x *g* for 30 min at 20 °C according to the method of Guo *et al.* (*3*). Particle size distribution in soymilk was analyzed with a laser particle size analyzer (LS230; Coulter, FL, U.S.A.).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Examining the Distribution of Lipids and Proteins in Soymilk

The soymilk was separated into the particulate, supernatant, and floating fractions in the similar manner as described above. Each fraction was frozen in liquid nitrogen and freeze-dried using a Freeze-dryer FD-1 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at -50 °C. The solids content was calculated by measuring the weight before and after freeze-drying. The freeze-dried samples contained little moisture (about 0.01%). The lipid content of each fraction was estimated by measuring the weight on a dry basis before and after defatting with n-hexane. The protein content after defatting was estimated using a modified Lowry assay (*DC* protein assay; Bio-Rad Laboratories, CA, U.S.A.) according to the manufacturer's instructions.

Fraction and Analyzing the Components of Precipitate after Adding $MgCl_2$

Soymilk was placed on ice for 1 h before the addition of $MgCl_2$. After the addition of $MgCl_2$ and placement on ice for 15 min, the ice-cold soymilk was separated into precipitate, supernatant, and floating fractions by centrifugation at 20,000 x *g* for 20 min at 4°C. The precipitate was freeze-dried, and the solids were measured. The protein composition of the precipitate was analyzed by SDS-PAGE, as described above. The same amount of defatted precipitate was electrophoresed, and the amounts of each protein were evaluated by stain intensity with CBB G-250. The TAG content in precipitate was evaluated by measuring the TAG contents in the soymilk, supernatant, and floating fractions using a Triglyceride E-Test Wako kit (Wako Pure Chemical Industries, Osaka). The TAG content in the precipitate was evaluated as follows:

$$\text{TAG (precipitate)} = \text{TAG (soymilk)} - \text{TAG (supernatant)} - \text{TAG (floating)}$$

Western Blotting

To detect the oleosin protein, Western blot analysis was performed using anti-oleosin antiserum. The antiserum was generated using a synthetic peptide antigen, EVGQKTKEVGQDIQC. The sequence EVGQKTKEVGQDIQ corresponds to residues 164-177 of oleosin A and 163-176 of oleosin B. Cysteine was added at the C-terminal end for coupling to keyhole limpet hemocyanin for antibody production and binding to an affinity column for purification of the antibody. Proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA), and then, after blocking with 10% skimmed milk in phosphate-buffered saline with 0.05% Tween 20, they were incubated with anti-oleosin antiserum and detected with secondary antibodies (goat anti-mouse IgG) using the Immun-Star Chemiluminescent Protein Detection Systems (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Measurement of Mineral Components in Soymilk

Calcium content in the soymilk was measured by atomic absorption spectrometry (Hitachi 170-30 atomic absorption spectrophotometer; Hitachi, Ltd., Tokyo, Japan) according to the method of Ono and Odagiri (*15*). The soymilk was diluted to 2.5%. Then 1000 ppm of strontium was added to suppress interference by phosphorus (*16*). A calcium standard solution (Kanto Chemical Co., Inc., Tokyo, Japan) was used as the standard. The other minerals were measured using an ICP atomic emission spectrometer (OPTIMA4300DV; PerkinElmer Japan Co., Ltd., Yokohama, Japan). The soymilk was dried by heating it in an oven at 105 °C and then dissolved in 1 N HCl.

Preparing the Dialyzed Extracts of Okara

The okara obtained after filtering the homogenate were washed with distilled water and dispersed in a 50 mM KCl solution. Extracts were obtained by boiling the okara in the 50 mM KCl solution in a water bath for 5 min above 95 °C and filtering through a defatted cotton sheet. Filtered extracts were dialyzed using a dialysis membrane having an exclusion size of 10 kDa, and freeze-dried using a Freeze-dryer FD-1 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) in the similar manner to the "Examining the distribution of lipids and proteins in soymilk" section.

Statistical Analysis

Data on the 11S/7S ratio and $MgCl_2$ concentrations for the maximum breaking stress were obtained from at least two individual experiments, and were analyzed with the statistical package SPSS 12.0 (SPSS, Chicago, IL).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Results and Discussion

Effects of Protein Component on Behavior of Lipids and Proteins and Coagulation Reactivity

Change in Distribution of Lipids and Proteins

Soymilk was fractionated at 20,000 x g after the addition of $MgCl_2$ using four varieties, Yumeminori, Fukuyutaka, Sachiyutaka, and Tosan 205, grown in 2005 in the field at our institute (Ibaraki, Japan), and the distribution of lipids and proteins was studied. Among the five soybean varieties examined, Fukuyutaka showed the highest 11S/7S ratio and the lowest $MgCl_2$ concentrations for the maximum breaking stress of tofu (*17*). In contrast, Sachiyutaka showed a relatively low 11S/7S ratio and the highest $MgCl_2$ concentrations for the maximum breaking stress. Yumeminori lacked the α and α' subunits of β-conglycinin, and Tosan 205 lacked all subunits of glycinin. The precipitate after centrifugation at 20,000 x g increased as greater concentrations of $MgCl_2$ were added (Figure 2). The precipitate probably consisted of protein and lipids which form particles. Precipitation took place at lower concentrations of $MgCl_2$ for Yumeminori, Fukuyutaka, Sachiyutaka, and Tosan 205 in that order, indicating that the increase in the precipitate was accelerated by increasing the 11S/7S ratio.

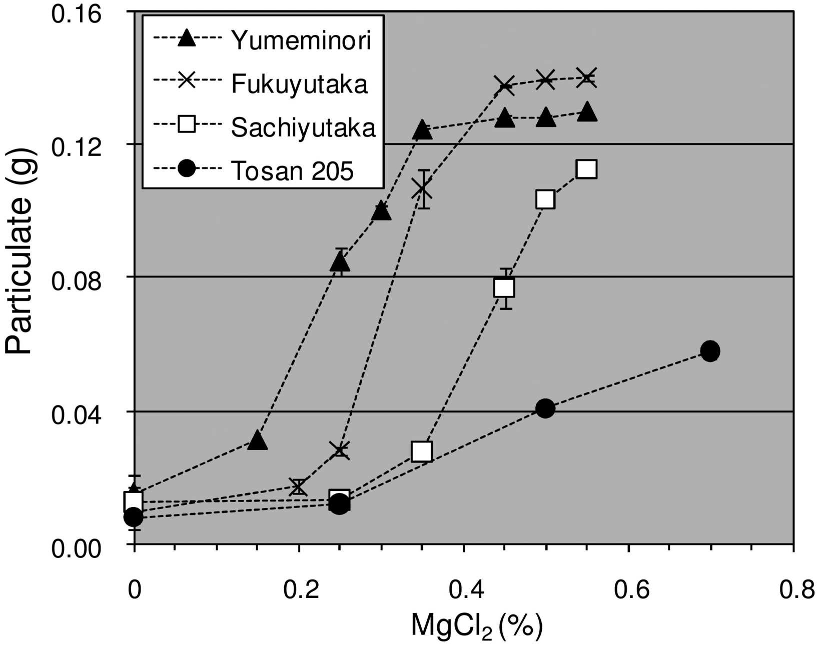


Figure 2. Changes in the amount of precipitate of soymilk after the addition of various concentrations of $MgCl_2$. ▲, x, □ and • represent the plots for Yumeminori, Fukuyutaka, Sachiyutaka and Tosan 205 respectively.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

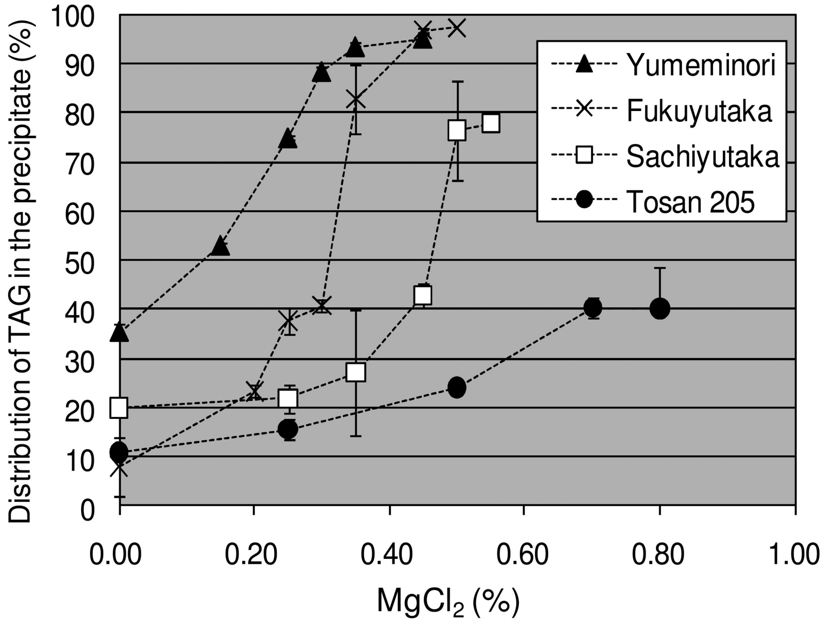


Figure 3. Changes in the distribution of TAG in the precipitate with addition of various concentrations of $MgCl_2$. ▲, x, □ and • represent the plots for Yumeminori, Fukuyutaka, Sachiyutaka and Tosan 205 respectively.

The distribution of TAG, a major component of soybean lipids, in the precipitate was examined after adding various concentrations of $MgCl_2$ (Figure 3). Guo *et al.* (*3*) reported that most of the lipid in unheated soymilk was in the particulate fraction, and was liberated, shifting to the floating fraction, after heating. In this study, we used heated soymilk ("nama-shibori" soymilk), and a small portion of TAG was detected in the precipitate at 0% $MgCl_2$ for Fukuyutaka, Sachiyutaka, and Tosan 205, which agrees with the report by Guo *et al.* (*3*). In contrast, about one-third of the TAG in the soymilk was detected in the precipitate at 0% $MgCl_2$ for Yumeminori. Since the same amount of precipitate was detected at 0% $MgCl_2$ for the four varieties (Figure 2), it is to be concluded that a larger amount of the lipid interacts with proteins in the precipitate of Yumeminori than in those of the other varieties. TAG in the precipitate was also increased by increasing the $MgCl_2$ concentration or the 11S/7S ratio of soybeans (Figure 3). These results indicate that as the $MgCl_2$ concentration or the 11S/7S ratio of soybeans increased, the incorporation of lipids and proteins in the precipitate increased.

The Protein Composition of the Precipitate at Various Concentrations of $MgCl_2$

The coagulum at the first step of the coagulation reaction probably consists of the precipitate observed in this study. The amount and composition of the precipitate may be an important factor in the coagulation reaction. The protein composition of the precipitate at various concentrations of $MgCl_2$ for the four varieties was studied by SDS-PAGE (Figure 4). CBB staining showed that about 80% of total proteins in the precipitate were glycinin, β-conglycinin, and oleosin at each $MgCl_2$ concentration for Yumeminori, Fukuyutaka, and Sachiyutaka, while about 45% of total proteins in the precipitate were β-conglycinin and oleosin for Tosan 205. Oleosin is a membrane protein of oil body (*18*).

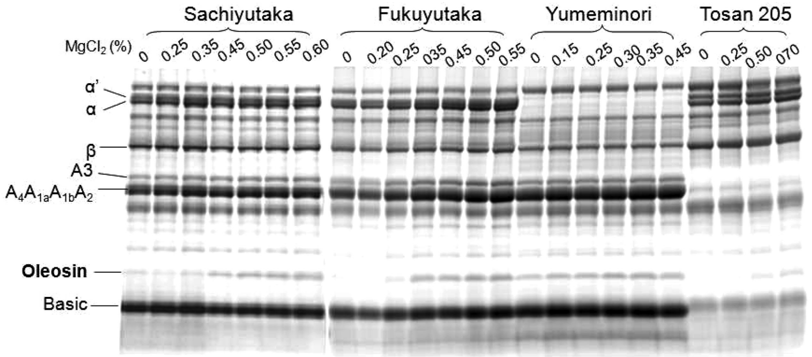

Sachiyutaka
Fukuyutaka
Yumeminori
Tosan 205
MgCl2 (%)
0
0.25
0.35
0.45
0.50
0.55
0.60
0
0.20
0.25
035
0.45
0.50
0.55
0
0.15
0.25
0.30
0.35
0.45
0
0.25
0.50
070
α'
α
β
A3
A4A1aA1bA2
Oleosin
Basic

Figure 4. SDS-PAGE patterns of proteins in the precipitate after the addition of various concentrations of $MgCl_2$ for Yumeminori, Fukuyutaka, Sachiyutaka, and Tosan 205. The main protein subunits and oleosin are indicated by the arrows to the left of the lanes. The varieties and final concentrations of $MgCl_2$ added to the soymilk are indicated above the lanes.

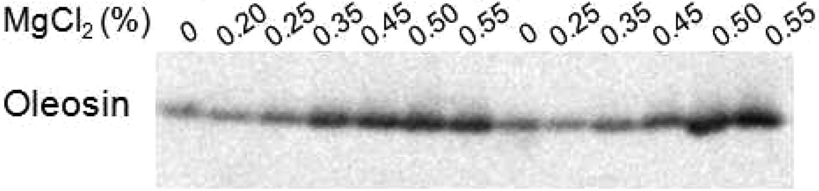

Fukuyutaka
Sachiyutaka
MgCl2 (%)
0
0.20
0.25
0.35
0.45
0.50
0.55
0
0.25
0.35
0.45
0.50
0.55
Oleosin

Figure 5. Western blot analysis using anti-oleosin antiserum. The same amount of proteins was electrophoresed as in Fig. 5 and blotted onto the PVDF membrane. The varieties and final concentrations of $MgCl_2$ added to the soymilk are indicated above the lanes.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

The stain intensity of the proteins indicated that the oleosin band increased as the $MgCl_2$ concentration increased for all four varieties, while the glycinin and the β-conglycinin bands were less variable at various $MgCl_2$ concentrations within the same variety (Figure 4 and Table 1). The oleosin band became more visible at lower concentrations of $MgCl_2$ for Yumeminori, Fukuyutaka, Sachiyutaka, and Tosan 205 in that order. A comparison of Yumeminori and Tosan 205 showed markedly different band intensities at the same $MgCl_2$ concentration (Table 1). There was little discernible difference between Fukuyutaka and Sachiyutaka. The difference was studied by Western blot analysis using anti-oleosin antiserum (Figure 5). Bands were more visible at lower concentrations of $MgCl_2$ for Fukuyutaka than for Sachiyutaka.

The present study indicates that the coagulum at the first step of coagulation increases as the 11S/7S ratio or the coagulant concentration increases (Figure 2). In addition, since oleosin is a membrane protein of oil bodies, a major component of oil globules (*3*, *18*), the results of SDS-PAGE (Figures 4), Western blot analysis (Figure 5), and changes in the distribution of TAG (Figure 3) indicate that as the 11S/7S ratio or the coagulant concentration increase, the concentration of oil globules in the coagulum increases. A single oil body was about 0.4 μm in diameter (*19*) making it much larger than the protein particles in soymilk, which were typically less than 100 nm in diameter (*20*). Many studies have reported on the relationship between lipids and the texture profile of soybean protein-lipid-water gel (*21–23*). The concentration of the oil globules in the coagulum might have a major effect on the formation of tofu curd. Therefore the 11S/7S ratio in soybeans and the coagulant concentration might have an effect on the amount and lipid concentration of the coagulum at the first step of coagulation, which in turn might affect the texture of tofu. Observation of tofu structure using an electron microscopy has revealed that the tofu microstructure was changed by changing 11S/7S ration of soybean and GDL or calcium concentrations as coagulants (*24*), which supports our idea.

Variation in the 11S/7S Ratio and the Coagulation Reactivity of Soymilk

The $MgCl_2$ concentrations for the maximum breaking stress of tofu ranged from 0.30% to 0.50% for the soybeans of the five Japanese varieties. The $MgCl_2$ concentrations for the maximum breaking stress showed a significantly negative correlation with the 11S/7S ratio in soybeans for all five Japanese varieties ($n = 47$, $r = -0.77$, $p < 0.001$, Figure 6). These results indicate that variation in the 11S/7S ratio has a major effect on the coagulation reactivity of soymilk, probably due to a change of physicochemical properties of soymilk.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Table 1. Protein Band Intensities in Precipitate after SDS-PAGE and CBB G-250 Staining

	Yumeminori						*Fukuyutaka*							*Sachiyutaka*							*Tosan 205*			
$MgCl_2$ (%)	0.00	0.15	0.25	0.30	0.35	0.45	0.00	0.20	0.25	0.35	0.45	0.50	0.55	0.00	0.25	0.35	0.45	0.50	0.55	0.60	0.00	0.25	0.50	0.70
Proteins																								
α'							0.35	0.24	0.28	0.43	0.46	0.49	0.37	0.13	0.16	0.19	0.24	0.26	0.37	0.23	0.26	0.33	0.42	0.37
α							0.35	0.29	0.33	0.42	0.46	0.63	0.53	0.32	0.49	0.59	0.47	0.47	0.38	0.41	0.31	0.35	0.32	0.35
β	0.22	0.22	0.22	0.21	0.22	0.22	0.47	0.56	0.42	0.44	0.44	0.49	0.38	0.42	0.47	0.49	0.42	0.42	0.43	0.42	0.62	0.81	0.63	0.61
A_3	0.15	0.15	0.17	0.16	0.20	0.20	0.22	0.25	0.30	0.31	0.32	0.38	0.35	0.15	0.17	0.20	0.19	0.20	0.22	0.24				
$A_4A_{1a}A_{1b}A_2$	0.81	0.95	0.89	0.86	1.02	1.07	0.86	0.99	1.14	1.20	1.27	1.44	1.30	0.76	0.87	0.97	0.88	0.92	0.92	0.96				
Oleosin	**0.07**	**0.10**	**0.09**	**0.10**	**0.12**	**0.14**	**0.04**	**0.04**	**0.07**	**0.16**	**0.15**	**0.18**	**0.16**	**0.05**	**0.03**	**0.06**	**0.14**	**0.17**	**0.19**	**0.19**	**0.02**	**0.03**	**0.05**	**0.06**
Basic	1.18	1.44	1.20	1.18	1.36	1.52	1.00	1.06	1.15	1.12	1.15	1.29	1.11	1.11	1.16	1.23	1.07	1.13	1.12	1.17				

The band intensities were evaluated in comparison with the intensity of the basic subunit of Fukuyutaka at 0% $MgCl_2$ on the same acrylamide gel, which was standardized as 1.00.

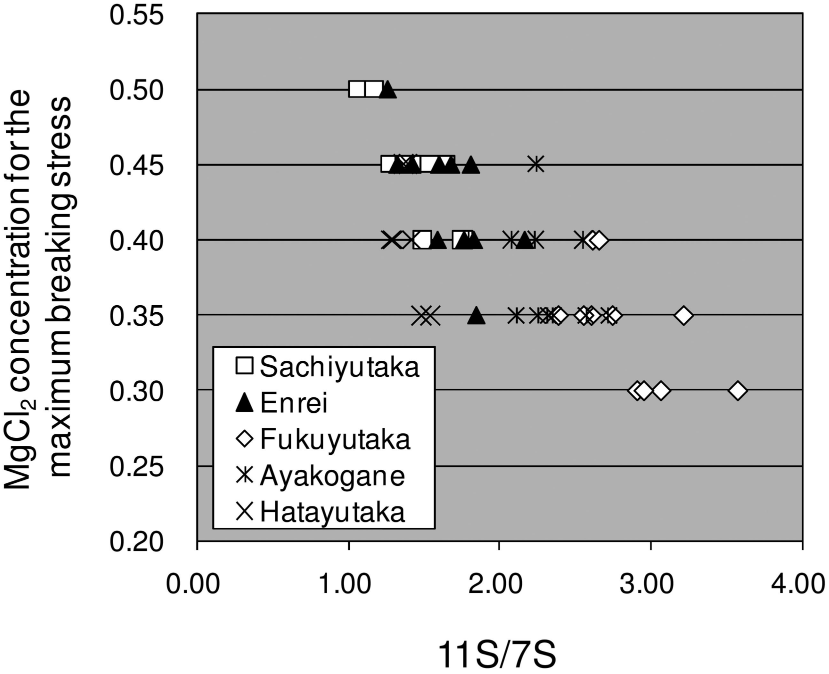


Figure 6. Correlation between 11S/7S ratio and $MgCl_2$ concentrations for maximum breaking stress of tofu for five Japanese varieties, Fukuyutaka, Enrei, Sachiyutaka, Ayakogane and Hatayutaka.

Effects of Other Components on Physichochemical Properties of Soymilk

Differences in Content of Particulate Protein and Size Distribution of Particles

Methods of making soymilk are roughly grouped into two kinds, “nama-shibori” and “kanetsu-shibori”. In the “nama-shibori” method, which is used mainly in Okinawa and Asian countries other than Japan, raw soymilk is processed by squeezing the slurry and then heating. In contrast, soymilk is obtained by squeezing the slurry after heating in the “kanetsu-shibori” method, which is used by most tofu manufacturers in Japan. We focused on the components from okara, which may be easier to extract in heated slurry using the “kanetsu-shibori” method. (see materials and methods). The relative particulate protein content was higher in soymilk prepared by the “semi-kanetsu-shibori” method, in which 62% of the protein was precipitated, while only 51% of the protein was precipitated in soymilk prepared by the “nama-shibori” method, on average (Table 2).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Table 2. Particulate Protein Content, Breaking Stress of Tofu and pH for the "Nama-shibori" Soymilk, the "Semi-kanetsu-shibori" Soymilk, and the "Nama-shibori" Soymilk with Added Calcium Chloride

	Nama-shibori	*Semi-kanetsu-shibori*	*+ 0.5 mM Ca (before heating)* †	*+ 2.0 mM Ca (before heating)* †	*+ 0.5 mM Ca (after heating)* ‡	*+ 2.0 mM Ca (after heating)* ‡
Particulate protein (%)	51^{b}	62^{a}	56	56	57	58
Breaking stress of tofu (kPa)	12.8^{b}	14.6^{a}	11.7^{b}	11.8^{b}	13.7	13.5
pH	6.77^{b}	6.61^{a}	6.59^{ab}	6.52^{ab}	6.57^{ab}	6.50^{ab}

[a] Significantly different value from that of the "nama-shibori" soymilk ($p<0.05$).

[b] Significantly different value from that of the "semi-kanetsu-shibori" soymilk ($p<0.05$).

† Calcium was added before heating soymilk.

‡ Calcium was added after heating soymilk.

Tofu was made by adding 0.3% GDL for all soymilk samples.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

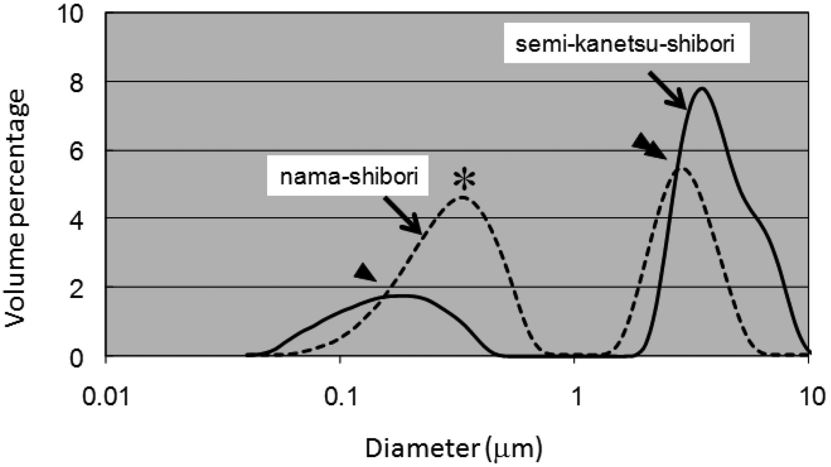


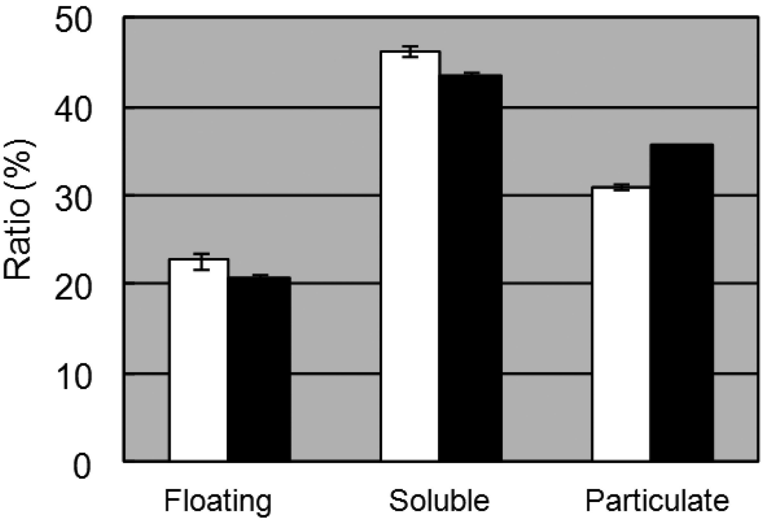
Figure 7. Particle size distribution (on volume percentage basis) for the "nama-shibori" soymilk (dotted line) and the "semi-kanetsu-shibori" soymilk (solid line). The asterisk indicates the peak of 370 nm in diameter, which is supposed to be that of oil bodies. The arrowhead and double arrowhead indicate positions of 150 nm and 3 µm in diameter, respectively.

To clarify whether the change in size distribution occurs along with the change in the particulate protein content, the distribution of the size of the particles was estimated using a laser particle size analyzer (Figure 7). In comparison with the "nama-shibori" soymilk, from 40 nm to 150 nm in diameter, the volume percentage bases increased in the "semi-kanetsu-shibori" soymilk, which suggests an increase of smaller protein particles. Protein particles larger than 40 nm in soymilk were revealed to interact by S-S bonding because they disappeared with the addition of 2-mercaptoethanol or Na-ascorbate (*20*). From 150 nm to 3.0 µm in diameter, the volume percentage bases decreased, followed by an increase in diameter of more than 3.0 µm in the "semi-kanetsu-shibori" soymilk. The peak at around 370 nm of the "nama-shibori" soymilk may represent particles made of a single oil body (*19*). These observations indicate that compared with the soymilk prepared by the "nama-shibori" method, the protein quantity in particles increased and some of particles interacted with oil bodies to form particles of larger size in the soymilk prepared by the "semi-kanetsu-shibori" method.

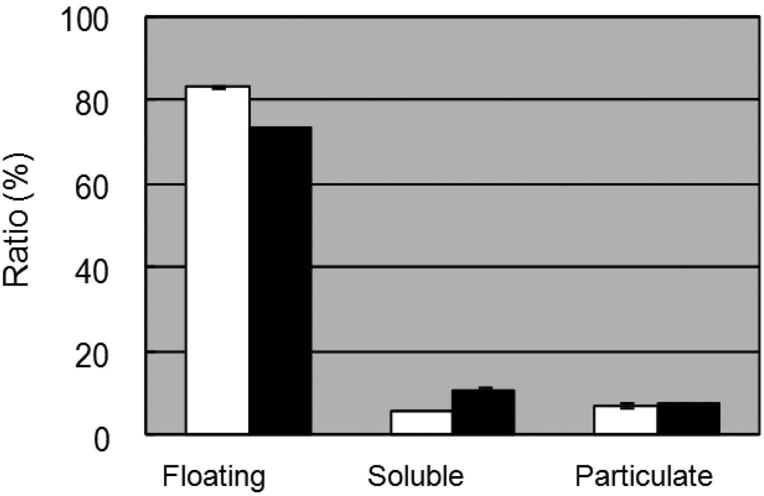
To examine the distribution of lipids, soymilk was fractionated into particle, soluble, and floating fractions by centrifugation and the distribution of solids, and the contents of lipids and proteins in solids were measured for each fraction (Figure 8). The solids contents of the soymilk were similar, about 9.5%, in both methods. The distribution of solids to the particulate fraction increased by 5% while those to other fractions decreased in the soymilk prepared by the "semi-kanetsu-shibori" method (Figure 8a), in accordance with the increase in the relative particulate protein content (Table 2).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

a



b



c

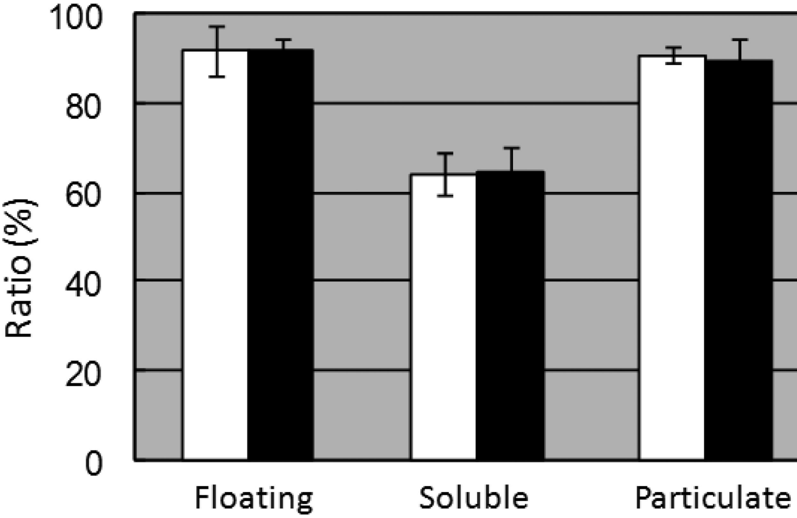


Figure 8. Distribution of solids (a), contents of lipids in solids (b), and contents of proteins in defatted solids (c) in floating, soluble, and particulate fractions for the "nama-shibori" soymilk (white bars) and the "semi-kanetsu-shibori" soymilk (black bars). The small bars at the top represent standard deviations.

The lipid content decreased in the solids of the floating fraction and increased in the solids of the soluble fraction by the "semi-kanetsu-shibori" method (Figure 8b). These observations indicate that oil bodies, which are major components of the floating fraction (*2*, *3*), became heavier to transfer from the floating fraction to the soluble fraction probably due to interaction with protein particles. This finding is also supported by the SDS-PAGE patterns of each fraction (Figure 9). The bands of 24 kDa and 18 kDa were found in the floating fraction of the soymilk prepared by the "nama-shibori" method, which is peculiar to this fraction (*2*). These two bands were revealed to be oleosin proteins by immunoblot. The intensities of these two bands were reduced in the floating fraction of the soymilk prepared by the "semi-kanetsu-shibori" method, indicating the transfer of oil bodies from this fraction. The lipid content in the solids and the protein content in the defatted solids for the particulate fraction was not different (Figures 8b and 8c), indicating that the same ratio of proteins to lipids was precipitated in this fraction for the soymilk of both methods. Since the distribution of solids to the particulate fraction increased (Fig. 8a), these findings provide supporting evidence for the change of distribution of lipids and the increase of the amount of both proteins and lipids in the particulate fraction in the "semi-kanetsu-shibori" soymilk.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

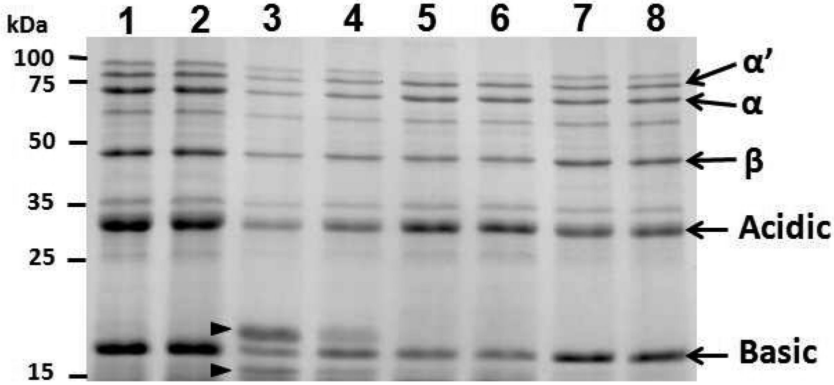


Figure 9. SDS-PAGE patterns of the soymilk (lanes 1 and 2), floating fractions (lanes 3 and 4), soluble fractions (lanes 5 and 6), and particulate fractions (lanes 7 and 8) for the "nama-shibori" soymilk (lanes 1, 3, 5, and 7) and the "semi-kanetsu-shibori" soymilk (lanes 2, 4, 6, and 8). Size markers are to the left of the lanes. The small triangles represent bands of 24 kDa and 18 kDa oleosin proteins, respectively. Main protein subunits are indicated by the arrows to the right of the lanes.

Changes in the Mineral Content and Their Effect on the Physicochemical Properties of Soymilk and on the Texture Profile of Tofu

Major mineral components such as calcium, potassium, and magnesium were increased in soymilk prepared by the "semi-kanetsu-shibori" method (Table 3). Calcium and magnesium were increased by 0.53 mM and 0.82 mM, respectively. According to the Standard Tables of Food Composition in Japan (fifth revised edition, Resources Council, Science and Technology Agency, Japan, 2000), okara includes 0.35% potassium, 0.08% calcium and 0.04% magnesium. About 20% of total magnesium and 45% of total calcium in seeds remained in okara, which were higher than that of protein (*25*). The divalent ions such as calcium and magnesium are believed to interact with pectin in seed coats (*26*), most of okara derives from. More minerals may be extracted by the "semi-kanetsu shibori" method than by the "nama-shibori" method. Since calcium and magnesium can act as coagulants and their effects are likely to be similar, the effects of calcium on pH, particulate protein content, and breaking stress of tofu made with 0.30% GDL were studied by adding 0.5 mM or 2.0 mM calcium chloride to the "nama-shibori" soymilk (Table 1). As a result, similar events as "semi-kanetsu-shibori" soymilk were observed except the breaking stress of tofu made from soymilk with addition of calcium before heating. The effect of calcium was more significant when it was added after heating than before heating for all parameters. The difference between the two treatments may partly due to the differences in the denatured state of proteins which interact with calcium. Calcium added after heating may function mainly as a coagulant, while calcium added before heating may interact with protein before denaturation, which occurs in "semi-kanetsu-shibori" soymilk.

Table 3. Content (mM) of Calcium, Potassium, and Magnesium in the "Nama-shibori" Soymilk and the "Semi-kanetsu-shibori" Soymilk

	Nama-shibori	*Semi-kanetsu-shibori*
Calcium	2.82	3.36
Potassium	41.32	49.85
Magnesium	11.43	12.25

Particulate protein content and the breaking stress of tofu increased particularly when calcium was added after heating, but the values were not significantly different from those of the "nama-shibori" soymilk. These results imply an effect of components other than calcium and magnesium. The content of phytic acid, which was revealed to play an important role in pH reduction and coagulation reaction (*4*, *5*), was not significantly different (data not shown).

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Changes in the Components of Macromolecules and Their Effect on the Physicochemical Properties of Soymilk and Texture Profile of Tofu

To study the effect of components other than minerals, the extracts of okara were dialyzed using a dialysis membrane, freeze-dried, and added to the "nama-shibori" soymilk before heating (see Materials and Methods). Table 4 shows the effect of the dialyzed extracts on particulate protein content, and breaking stress of tofu made with 0.30% GDL. The "semi-kanetsu-shibori" soymilk is estimated to contain 0.044% dried extracts. The particulate protein content also increased as greater amounts of the dialyzed extracts were added. With the addition of 0.025% of the dialyzed extracts, particulate protein content reached nearly the same value as that of the "semi-kanetsu-shibori" soymilk. The breaking stress of tofu also increased, but showed a peak with the addition of 0.025% of the dialyzed extracts, and decreased with the addition of greater amounts of the extracts, even though the particulate protein content was still increasing. Since the breaking stress of tofu made with 0.30% GDL increased in a previous study when the particulate protein content increased from 35.6% to 78.3% (*27*), the decrease of the breaking stress of tofu in this study is unlikely to have been due to the excessive amount of particles.

We observed that after centrifugation, lipids in the floating fraction disappeared and precipitated in soymilk that contained 0.050% or 0.070% of the dialyzed extracts, which is different from the "nama-shibori" and the "semi-kanetsu-shibori" soymilk. Similar event could be observed when larger amount of a coagulant was added in soymilk; almost all TAG was precipitated at higher $MgCl_2$ concentrations (Figure 3). Excess amount of $MgCl_2$ also resulted in decrease in the breaking stress of tofu (Figure 1). Our data suggest that macromolecules of extracts from okara play an important role in increasing particulate protein content, behavior of proteins and lipids, and the breaking stress of tofu.

Table 4. Particulate Protein Content and Breaking Stress of Tofu for the "Nama-shibori" Soymilk, the "Semi-kanetsu-shibori" Soymilk, and the "Nama-shibori" Soymilk with Added Extracts of Okara

	Nama-shibori	*Semi-kanetsu-shibori*	*+ dialyzed extracts (0.010%)*	*+ dialyzed extracts (0.020%)*	*+ dialyzed extracts (0.025%)*	*+ dialyzed extracts (0.050%)*	*+ dialyzed extracts (0.070%)*
Particulate protein (%)	51^b	62^a	53^b	56^b	61^a	71^a	75^{ab}
Breaking stress of tofu (kPa)	12.8^b	14.6^a	13.0	13.9	14.2	10.2^{ab}	6.6^{ab}

[a] Significantly different value from that of the "nama-shibori" soymilk (p<0.05).
[b] Significantly different value from that of the "semi-kanetsu-shibori" soymilk (p<0.05).

To analyze the components of macromolecules, FT-IR analysis was performed (*28*). The spectrum of the dialyzed extracts and that of lemon pectin showed common peaks, one of which is 1750 cm^{-1} due to esterified carboxyl groups and the other is 1200 to 1000 cm^{-1}, common to saccharides (*29*). The spectrum of the dialyzed extracts was similar to that of pectin from soybean seed coats shown by Monsoor*et al.* (*30*). These facts indicate that the extracts contained polysaccharides.

The IR spectrum of the dialyzed extracts shows two more peaks of 1640^{-1} cm and 1545 cm^{-1}, which the IR spectrum of lemon pectin lacks. These peaks were likely due to amides of protein (*29*). The protein content of the extracts of okara was estimated to be 13.3%, on a dry basis, by a colorimetric assay. The SDS-PAGE patterns showed the main band of 27 kDa in the dialyzed extracts (Figure 10), corresponded to the subunit of Basic 7S globulin, which was revealed to be the main protein in extracts of okara in hot water (*31*). Basic 7S globulin is a cysteine-rich glycoprotein that has 27- and 16-kDa subunits linked by disulfide bonds (*32*), being localized mainly in the middle lamella of cell walls in cotyledons (*33*). It is likely that cysteine residues and sugar chains of Basic 7S globulin can interact with proteins, lipids, and polysaccharides to affect the physicochemical properties of soymilk. Protein particulate content was also increased after adding extracted Basic 7S globulin (data not shown) to soymilk. Further studies are required to reveal the effect of Basic 7S globulinon physichochemical properties of soymilk.

Difference in the Texture Profile of Tofu

The breaking stress of tofu coagulated with 0.30% GDL was measured for soymilk prepared by the "nama-shibori" and "semi-kanetsu-shibori" methods (Table 2). The breaking stress of the "semi-kanetsu-shibori" method was higher, which can be interpreted to mean that an increase in particulate protein results in an increase in the breaking stress of tofu, which agrees with the report by Guo and Ono (*27*). Increase in breaking stress of tofu may primarily due to change in the number and size of protein particles.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

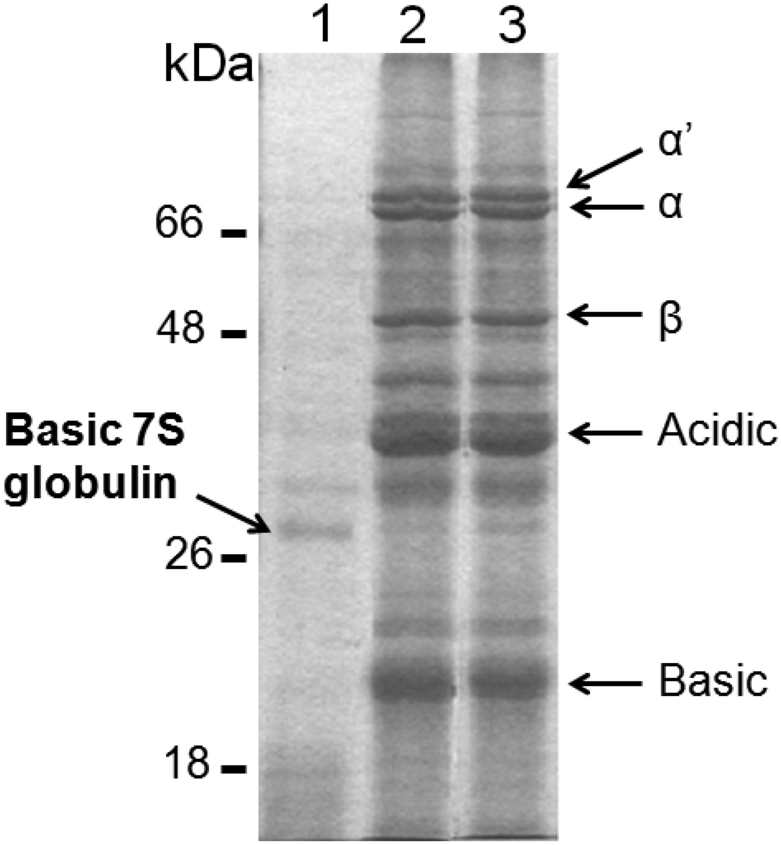


Figure 10. SDS-PAGE patterns of the dialyzed extracts of okara (lane 1), the "nama-shibori" soymilk (lane 2), and the "semi-kanetsu-shibori" soymilk (lane 3). Size markers are to the left of the lanes. Main protein subunits are indicated by the arrows.

"Semi-kanetsu-shibori" soymilk also showed higher viscosity than "nama-shibori" soymilk (*28*), which is probably due to difference in components described above. Although viscosity may partly affect the breaking stress of tofu, the main problem about high viscosity is probably lower extraction efficiency and fluidity. They are key factor for handling. Viscosity may become as important as the breaking stress of tofu concerning the processing properties of tofu.

Acknowledgments

This study was supported financially by the Kakou-Puro project of the Ministry of Agriculture, Forestry, and Fisheries of Japan.

References

1. Ono, T.; Katho, S.; Mothizuki, K. Influences of calcium and pH on protein solubility in soybean milk. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 24–28.
2. Guo, S. T; Ono, T.; Mikami, M. Interaction between protein and lipid in soybean milk at elevated temperature. *J. Agric. Food Chem.* **1997**, *45*, 4601–4605.
3. Guo, S. T.; Ono, T.; Mikami, M. Incorporation of soy milk lipid into protein coagulum by addition of calcium chloride. *J. Agric. Food Chem.* **1999**, *47*, 901–905.
4. Tezuka, M.; Taira, H; Igarashi, Y; Yagasaki, K.; Ono, T. Properties of tofus and soy milks prepared from soybeans having different subunits of glycinin. *J. Agric. Food Chem.* **2000**, *48*, 1111–1117.
5. Ono, T. Associates of protein in milk and soymilk. *New Food Ind.* **1999**, *41*, 65–78, in Japanese.
6. Yamauchi, F.; Yamagishi, T.; Iwabuchi, S. Molecular understanding of heat-induced phenomena of soybean proteins. *Food Rev. Int.* **1991**, *7*, 283–322.
7. Saio, K.; Kamiya, M.; Watanabe, T. Food processing characteristics of soybean 11S and 7S protein. Part I. Effect of difference of protein component among soybean varieties on formation of tofu-curd. *Agric. Biol. Chem.* **1969**, *33*, 1301–1308.
8. Guo, S. T.; Ono, T. The role of composition and content of protein particles in soymilk on tofu curding by glucono-δ-lactone or calcium sulfate. *J. Food Chem.* **2002**, *70*, C258–C262.
9. Guo, S. T.; Tsukamoto, C.; Takahashi, K; Yagasaki, K.; Nan, Q. X.; Ono, T. Incorporation of soymilk lipid into soy protein coagulum by the addition of calcium chloride. *J. Food Sci.* **2002**, *67*, 3215–3219.
10. Liu, Z. S.; Chang, S. K. C. Effect of soymilk characteristics and cooking conditions on coagulant requirements for making filled tofu. *J. Agric. Food Chem.* **2004**, *52*, 3405–3411.
11. Liu, Z. S.; Chang, K. C. Development of a rapid titration method for predicting optimal coagulant concentration for filled tofu. *J. Agric. Food Chem.* **2003**, *51*, 5214–5221.
12. Toda, K.; Ono, T.; Kitamura, K.; Hajika, M.; Takahashi, K.; Nakamura, Y. Seed protein contents and consistency of tofu made with different magnesium chloride concentrations in six Japanese soybean varieties. *Breed. Sci.* **2003**, *53*, 217–223.
13. Yagasaki, K.; Kaizuma, N.; Kitamura, K. Inheritance of glycinin subunits and characterization of glycinin molecules lacking the subunits in soybean (*Glycine max* (L.) Merr). *Breed. Sci.* **1995**, *46*, 11–15.
14. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
15. Ono, T.; Odagiri, S. Determination of calcium in milk by atomic absorption spectroscopy. *Jap. J. Dairy Sci.* **1975**, *24*, A 133–138.
16. Willis, J. B. Determination of calcium and magnesium in urine by atomic absorption spectroscopy. *Anal. Chem.*, *33*, 556–559.
17. Toda, K.; Yagasaki, K.; Takahashi, K. Relationship between protein composition and coagulation reactivity, particulate formation, and incorporation of lipids in soymilk. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 2824–2830.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

18. Tzen, J. T. C.; Lai, Y. K.; Chan, K. L.; Huang, A. H. C. Oleosin isoforms of high and low molecular weights are present in the oil bodies of diverse seed species. *Plant Physiol.* **1990**, *94*, 1282–1289.
19. Huang, A. H. C. Oil bodies and oleosin in seeds. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 177–200.
20. Ono, T.; Myoeng, R. C.; Ikeda, A.; Odagiri, S. Changes in the composition and size distribution of soymilk protein particles by heating. *Agric. Biol. Chem.* **1991**, *55*, 2291–2297.
21. Catsimpoolas, N.; Meyer, E. W. Gelation phenomena of soybean globules. III. Protein-lipid interactions. *Cereal Chem.* **1971**, *48*, 159–167.
22. Shimada, K.; Matsushita, S. Effect of oils on thermal gelation of soybean protein. *Agric. Biol. Chem.* **1981**, *45*, 2877–2881.
23. Miura, M.; Yamauchi, F. Rheological behavior of soybean protein-lipid-water gel, from a small distortion to rupture. *Agric. Biol. Chem.* **1984**, *48*, 2449–2455.
24. Onodera, Y.; Ono, T.; Nakasato, K.; Toda, K. Homogeneity and microstructure of tofu depends on 11S/7S globulin ratio in soymilk and coagulant concentration. *Food Sci. Technol. Res.* **2009**, *15*, 265–274.
25. Nakayama, O.; Fukamachi, C.; Kubo, S.; Suruga, S.; Miyanaga, S.; Horiie, S.; Watanabe, A. *Rep. Res. Inst.* **1960**, *14B*, 52–55, in Japanese.
26. Ridley, B. L; O'Neill, M. A; Mohnen, D. Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **2001**, *57*, 929–967.
27. Guo, S. T.; Ono, T. The role of composition and content of protein particles in soymilk on tofu curding by glucono-δ-lactone or calcium sulfate. *J. Food Chem.* **2005**, *70*, 258–262.
28. Toda, K.; Chiba, K.; Ono, T. Effects of components extracted from okara on the physicochemical properties of soymilk and tofu texture. *J. Food Sci.* **2007**, *72*, C108–113.
29. Nakasato, K.; Ono, T.; Ishiguro, T.; Takamatsu, M.; Tsukamoto, C.; Mikami, M. Rapid quantitative analysis of the major components in soymilk by using fourier-transform infrared spectroscopy (FT-IR). *Food Sci. Technol. Res.* **2004**, *10*, 137–142.
30. Monsoor, M. A.; Kalapathy, U.; Proctor, A. Determination of polygalacturonic acid content in pectin extracts by diffuse reflectance Fourier transform infrared spectroscopy. *Food Chem.* **2001**, *74*, 233–238.
31. Asano, M.; Endo, I.; Yamauchi, F. Soybean Basic 7S globulin which was detected from okara and its isolation methods. *Nippon Shokuhin Kogyo Gakkaishi* **1993**, *40*, 323–330in Japanese with English summary.
32. Kagawa, H.; Hirano, H. Sequence of a cDNA encoding soybean basic 75 globulin. *Nucleic Acids Res.* **1989**, *17*, 8868.
33. Nishizawa, N. K.; Mori, S.; Watanabe, Y.; Hirano, H. Ultrastructural localization of the Basic 7S globulin in soybean (*Glycine max*) cotyledons. *Plant Cell Physiol.* **1994**, *35*, 1079–1085.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch017

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Chapter 18

Development and Quality of Tofu Analogue Prepared from Whole Soybeans

Fuh-Juin Kao,[1] Nan-Wei Su,[2] and Min-Hsiung Lee*,[2]

[1]Hospitality Management Department, School of Tourism,
Ming Chuan University, Taoyuan, Taiwan
[2]Department of Agricultural Chemistry, National Taiwan University,
Taipei 10617, Taiwan
***mhlee@ntu.edu.tw**

Okara is the insoluble residue after filtration of soymilk. In common, every pound of dry beans makes into soymilk or tofu generates about 1.1 pounds of okara with around 80% moisture. It contains high content of fiber and appreciable amounts of oil and protein with high quality. However, okara is generally used as feed or fertilizer. With the growing awareness of the importance of dietary fiber in human health, there is an increasing interest in the utilization of whole beans as an alternative. In this study, a whole-soybean tofu was developed. Whole-soybean tofu treated with fine milling and with particle size smaller than 425 μm could be successfully made just by means of calcium sulfate addition. It was found the tofu made with water-to-bean ratio at 12:1 gave maximal protein and solid recoveries, as well as the maximal tofu yield. However, the whole-soybean tofu possessed softer, less chewy texture, and coarse appearance. Nevertheless, whole-soybean tofu was rich in fiber and low in fat. It could be considered as a healthy food.

Introduction

The soybean is one of the most valuable agricultural commodities because of its unique chemical composition. On the average, moisture content of stored mature soybeans is usually about 10-13%. On a dry basis, soybeans contain about 5-10% ash, 10% crude fiber, 16-22% crude fat, 40-50% crude protein, and about

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

20% carbohydrate (*1*). Besides, soybeans also contain various macronutrients such as isoflavones, its contents range from 1000-4000 ppm dependent on soybean variety (*2*). In the eastern Asia, soybeans had been transformed into forms of soyfoods, such as soymilk, tofu, soy sauce, and tempeh. However, tofu has been the most popular way to serve soybeans as a food. Many different types of tofu have appeared in the market. Based on the water content and textural properties, tofu is generally classified into Momen, Kinu, and packed tofu (*3*). Basically, these tofus are made in a similar fashion except for variations in the water-to-bean ratio, the type of coagulants used, and the amount of whey being pressed out. In Taiwan, momen tofu is subclassified into three basic types, namely regular, firm, and dry tofu according to its water content. In general, for regular tofu, the moisture content should be about 87-89%; for firm tofu, 76-87%; and for dry tofu, less than 76% (*4*, *5*).

During tofu preparation, the soaked beans are ground with fresh water and then the slurry is filtrated. The residue, known as soy pulp or okara is separated from the filtrate. Therefore, okara is considered a byproduct during soymilk or tofu preparation, most of it is dumped and burnt as waste. However, to avoid environmental consequences of its disposal, developing a reusing technique for okara is highly encouraged. In fact, dry okara generally contains protein up to 18.2-32.2% protein, 6.9-22.2% oil, 9.1-18.6% crude fiber (*6*). Not only is okara a rich source of dietary fiber, it also contains a high quality of protein. The okara protein is generally of higher quality than that obtained from other soy products both in terms of the protein efficiency ratio and the essential amino acid to total amino acid ratio (*7*). Notably, secondary metabolites such as isoflavones outstandingly serve as a healthy dietary supplement. According to the report of Jackson et al. (*8*), the estimated total mass of isoflavones lost in okara was 28% during tofu preparation. This compound is known to help preventing osteoporosis, breast and ovarian cancer, and cardiovascular diseases (*9*, *10*).

Several studies have investigated the use of the okara, including preparing protein isolate from okara by isoelectric precipitation (*11*), extracting emulsionizing polysaccharides from okara by hydrolysis (*12*) using okara as a base for growing *Bacillus subtilis* to produce iturin A-a fungicide effective against serious plant pathogens (*13*). .In terms of food application, there are various ways of using okara. In some parts of China, okara could be fermented with various microorganisms for the production of meitauza which is a natto-like or tempeh-like product (*7*, *14*). Basically, okara could be made into various types of food, including salad, sauces, bread (*15*), biscuit (*16*), and candy (*17*).

However okara putrefies very quickly because its high moisture content, to overcome this problem, *Chundubu* which is a kind of Korean whole-tofu made from microparticulated soybean powder, and no okara generated during *Chundubu* preparation (*18*). Although it offered the benefit of not creating the environmental problem, it was not popular with consumer for its rough texture. Afterward Ku et al. (*19*) try to use merceating enzymes to reduce the particle size of the whole soybean powder, however the effect on the reduction of particle size of okara was not significant. Therefore, the okara-containing-tofu could be developed to some tofu analogue, which is no need of smooth mouthfeel and appearance, such as Aburage or Kori-tofu. Aburage is produced by frying fresh tofu in oil, while Kori-

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

tofu is fresh tofu dehydrated through repeated freeze-thaw cycle, then turn into a sponge-like structure in the end. There are few reports about the method to make utilization of okara in tofu or its analogue, because the use of okara has been difficult, for the sake of high fiber content and unsavory texture for eating.

In this preliminary study, we tried to develop a process for making whole-soybean tofu with texture properties similar to the common tofu or its analogue and without okara generated during manufacturing process. The effects of preparation conditions such as okara powder size, water-to-bean ratio, and transglutaminase treatment on quality and textural properties of whole-soybean tofu were investigated.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Materials and Methods

Materials

Soybeans of the Ohio FG1 cultivar were obtained from a local agency. Food grade calcium sulfate dihydrate (Ako Kasei Co., Ltd. Hyogo, Japan) was used as the coagulant in the tofu production and was obtained from Gemfont Corporation (Taipei, Taiwan). Food grade antifoaming agent (containing 90% glycerin fatty acid ester, 5% calcium carbonate, 4.3% soybean phospholipids, and 0.7% silicone resin) was obtained from Riken Vitamin Co., Ltd. (Tokyo Japan). Microbial transglutaminase (**MTG**ase) preparation (0.044 U/mg) was a gift from Ajinomoto Co., Inc. The commercial **MTG**ase preparation consisted of 0.2% **MTG**ase, 60% sodium caseinate, and 39.8% maltodextrin.

Conventional Tofu Making

The procedure used for traditional tofu making was similar to that published by Cai and Chang (*20*) with some modifications. Aliquots of 300 g soybeans were soaked in tap water at 4°C for 9 h to bring the soaked beans weigh to approximately 2.2 times their initial weight.. After draining the initial soaking water, tap water was added to the hydrated beans to a final weight of 3300 g to give the water-to-bean ratios of 10:1. The mixtures were then ground with a soymilk grinder (Pineapple grinder, Great Yen Electric Food Grinder Co., Ltd. Taipei, Taiwan) equipped with an automatic centrifugal filter to separate raw soymilk from the residue. After grinding, the slurry was further filtrated through a 100-mesh sieve to remove the remaining okara. After adding 1 g of the antifoaming agent, soymilk was heated to boiling with gentle stirring and kept at boiling temperature (approximately 96 °C) for 5 min. After cooling to 73°C, 50 mL of calcium sulfate solution was added to the mixture to give the final $CaSO_4 \cdot 2H_2O$ concentration used in this mixture was 0.4% (w/v). Immediately after the addition of coagulant solution, soymilk milk was stirred at a speed of 250 rpm for 10 sec and then incubated for 20 min to form bean curd. The soybean curd was then broken slightly and transferred into a muslin cloth- lined stainless steel mold (13 x 13 x 5.5 cm^3) and then pressed at 21.8 g/cm^2 for 10 min, 43.6 g/cm^2 for 10 min, and 65.4 g/cm^2 for 30min. At the end of pressing, the cloth was removed, and the weight of tofu was recorded. The tofu yield was expressed as grams of tofu per 100 g of soybeans. The tofu was transferred into a plastic bag and stored at 4°C overnight for subsequent measurements.

Transglutaminase-Treated Tofu Making

The procedure used for transglutaminase-treated tofu making required some modifications, since the enzyme is not active above 70°C (*21*). Aliquots of 300 g soybeans were soaked in tap water at 4°C for 9 h to bring the soaked beans weigh to approximately 2.2 times their initial weight. After draining the initial soaking water, tap water was added to the hydrated beans to a final weight of 3300 g to give the water-to-bean ratios of 10:1. The mixtures were performed coarse grinding with a soymilk grinder (Pineapple grinder, Great Yen Electric Food Grinder Co., Ltd. Taipei, Taiwan) then put filtrated okara back into filtrated soymilk. After adding 1 g of antifoaming agent, the okara containing soymilk was heated to 96°C and held at this temperature for 5 min with constant stirring. The boiled okara containing soymilk was cooled to about 65°C , and then $CaSO_4 \cdot 2H_2O$ and the **MTG**ase suspended in 50 mL of water were added to this soymilk to give the final $CaSO_4 \cdot 2H_2O$ and **MTG**ase concentration used in this soymilk were 0.4% and 5.0 ppm, respectively (w/v). Immediately after the addition of coagulant and enzyme, the okara containing soymilk milk was stirred at speed of 250 rpm for 10 sec, and then incubated for 20 min to form okara containing bean curds. After the incubation, the curd was heated in a hot water bath (90°C) for 30 min to inactive the enzyme. This soybean curd was then broken slightly and transferred into a muslin cloth-lined stainless steel mold (13 x 13 x 5.5 cm^3) and pressed at 22 g/cm^2 for 10 min, 44 g/cm^2 for 10 min, and 65 g/cm^2 for 30 min. At the end of pressing, the cloth was removed, and the weight of okara containing tofu was recorded. The tofu yield was expressed as grams of fresh okara containing tofu per 100 g of soybeans. The okara containing tofu was transferred into a plastic bag and stored at 4°C overnight for subsequent measurements.

Whole Soybean Tofu Making

Three aliquots of 300 g soybeans were soaked in tap water (three times bean weight) at 4°C for 9 h to bring the soaked beans weigh to approximately 2.2 times their initial weight. After draining away the initial soak water, tap water was added to the hydrated beans to a final weight of 3300 g, 3900 g, and 4500 g to give water-to-bean ratios of 10:1, 12:1, and 14:1 (w/w), respectively. At first, the mixtures were performed coarse grinding with a soymilk grinder (Pineapple grinder, Great Yen Electric Food Grinder Co., Ltd., Taipei, Taiwan) and then put okara back into soymilk then followed by fine milling using a wet grinder with a sieve of 425 μm woven wire mesh (Super Fine Grinding machine, Tai Cheer Machinery Enterprise Co., Ltd., Taoyuan, Taiwan) to reduce okara particle size less then 425 μm. After adding 1 g of antifoaming agent, the okara containing soymilk was heated to 96°C and held at this temperature for 5 min with constant stirring. After cooling to 73°C, 50 mL of calcium sulfate solution was added to the mixture to give the final $CaSO_4 \cdot 2H_2O$ concentration used in this mixture were 0.4% (w/v), the mixture was stirred at a speed of 250 rpm for 10 sec, and then incubated for 20 min to form okara

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

containing bean curds. The soybean curd was then broken slightly and transferred into a muslin cloth-lined stainless steel mold (13 x 13 x 5.5 cm^3) and pressed at 22 g/cm^2 for 10 min, 44 g/cm^2 for 10 min, and 65 g/cm^2 for 30 min. At the end of pressing, the cloth was removed, and the weight of okara containing tofu was recorded. Tofu yield was expressed as grams of tofu per 100 g of soybeans. The okara containing tofu was transferred into a plastic bag and stored at 4°C overnight for subsequent measurements.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Determination of Moisture and Solid Content

The moisture content of the tofu and the total solid content of the soymilk were determined according to the procedure of the **AOAC** (*22*). Weighed quantities (4-5 g) of samples were oven-dried at 105°C until a constant weight was obtained.

Determination of Protein and Solid Contents and Their Recoveries

The protein contents in soybean and tofu were determined in triplicate by the micro-Kjeldahl method (*22*). The solid contents of soybean and tofu were determined by drying a 5 g homogenized sample in an oven at 105°C until constant weight was obtained. Protein recovery in tofu was expressed as the amount of protein in tofu divided by the amount of protein in raw soybean multiplied by 100% on a dry weight basis. The same calculation was applied to solid recovery.

Determination of Water Retention Ability (WRA) of Tofu

WRA was determined by a modification of the water holding capacity method (**WHC**) of Puppo and Añón (*23*). About 5 g (w_1) tofu was placed on a cotton cloth-membrane maintained in the middle position of a 250 mL centrifuge tube (62 mm × 120 mm). Recording the sample weight after centrifugation at 120 ×g for 5 min at 15°C (w_2) and subsequently heating to a constant weight (w_3) at 105°C. The **WRA** of tofu was calculated as following equation: **WRA**= ((w2-w3)/(w1-w3))×100%

Determination of Textural Properties

The textural properties of tofu were measured by a texture analyzer (Model TA-XT2, Stable Micro systems, Haslemere, Surrey, UK). A 5 kg load cell was used with the crosshead controlled at 1.5 mm/sec. A cylindrical plunger with a diameter of 35 mm was used to compress the tofu cakes (35 mm dia. × 22 mm ht.) to 50% deformation. Hardness, cohesiveness, gumminess, springiness, and chewiness were calculated using the Textural Profile Analysis curve (*24*). Hardness was defined as the height of the force peak on the first compression cycle, which was the force necessary to attain a given deformation. The ratio of the positive force areas during the second compression to that under the first compression was defined as cohesiveness. Gumminess was defined as the product of hardness and cohesiveness. The springiness was the degree of recovery to its original height after decompression and accordingly was expressed as the horizontal distance (mm) between the point when the second curve started and the point when the second curve reached a peak. Chewiness was defined as the product of gumminess and springiness. The data were means of 20 replicate measurements at room temperature.

Sensory Evaluation Test (Acceptance Test)

The panel was made up of thirty adult males and females who were lifelong tofu consumers and familiar with tofu products. The list of attributes selected by the authors as appropriate for defining the quality of the tofu are appearance, flavor, texture, and overall acceptability. However, the appearance attribute includes color, surface smoothness and homogeneity. The flavor attribute covers aroma and taste. The texture attribute contains firmness and elasticity. And the results were expressed on a 9-point hedonic scale. The sensory scores were 9, like extremely; 8, like very much; 7, like moderately; 6, like slightly; 5, neither like nor dislike; 4, dislike slightly; 3, dislike moderately; 2, dislike very much; 1, dislike extremely.

Samples for sensory evaluation were cut into cubic samples (2.0 cm) and cooked in boiling water for 5 min, then removed out and placed on sieves to drain followed by keeping at room temperature before evaluation. All samples were coded and always presented in a randomized arrangement.

Data Analysis

All results were analyzed by analysis of variance (**ANOVA**) using the general linear model (*25*). Duncan's multiple range test was used to determine differences among the samples. Significant levels were defined as probabilities of 0.05 or less. All processing treatments were in triplicate.

Results and Discussion

The Effects of Grinding-Treatment on Whole-Soybean Tofu Quality

Tofu of good quality is judged in terms of appearance, texture, aroma, taste and mouthfeel and high yield. Smoothness and texture are important attributes influencing acceptability of tofu by consumer. While, the yield of tofu is an important consideration for tofu manufactures to select processing conditions. Table 1 shows the effects of okara particle size on the quality of whole-soybean tofus prepared by coarse milling or fine milling (reduced okara particle size less then 425 µm). As compared with conventional tofu, the employ of okara caused significant increase in soymilk solid content, protein and solid recoveries for two kinds of whole-soybean tofu, hence, caused the increase in tofu yield. Regarding the increase in moisture content of tofu, it resulted from the high water binding ability of okara. Ma et al. (*26*) found that protein in okara possessed good water binding properties and was comparable to that of commercial protein source, in addition, polysaccharide in okara might be also responsible for the high water hydration ability of okara (*27*). However, the employ of okara significantly decreases the **WRA** of tofu (Table 1), result from okara destroys the uniformity of microstructure of tofu. In our previous studies (*28*) we suggested that the tofu

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

had the better uniform and homogeneous microstructure, resulted in the better WRA. In addition, Puppo and Añón (*23*), who also showed that a protein gel with a homogeneous and fine structure gave high **WRA** as compared to the gel with a nonhomogeneous structure, which had a high degree of syneresis. By the way, the whole-soybean tofu treated with coarse milling was not solidified by the $CaSO_4$. $2H_2O$, even if a gel matrix was formed, it was very fragile and tended to collapse when subject to slight shaking. However, the whole-soybean tofu treated with fine milling was observed that formed a stable and firm tofu gel after addition of calcium sulfate.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Analysis of Quality and Texture Properties for Transglutaminase Treated Tofu

The coagulation of soymilk is an important step during tofu manufacturing. Salts (e.g. $CaSO_4$, $CaCl_2$, $MgCl_2$ and $MgSO_4$) and acid (glucono-δ-lactone; **GDL)** have been used as the coagulant to prepare tofu. Besides those coagulants, **MTG**ase (EC .2.3.2.13) enzyme could also result in the coagulation of soymilk to produce tofu, and the formed tofu is more retort-resistant as compared with those tofus induced by other kinds of coagulants (*29*). **MTG**ase is reported that has ability to catalyze the formation of covalent cross-linking between soy proteins in soymilk, besides, Kwan and Easa (*30*) pointed out that tofu treated with **MTG**ase could reduce retort-induced syneresis of **GDL** tofu. Since the whole-soybean tofu treated with coarse-milling couldn't solidified by traditional coagulants, **MTG**ase was used to induce gel formation of this whole-soybean tofu. As shown in Table 2, **MTG**ase significantly increased **WRA** and protein recovery of "whole-soybean tofu" manufactured through coarse milling. Apparently, the coagulation of okara containing soymilk by means of **MTG**ase addition could increase the **WRA** and consequently result in low syneresis and less loss of soluble protein and other soluble substance. These events would seem to account for higher protein recovery.

Table 3 shows the impact of **MTG**ase on the textural properties of "whole-soybean tofu "treated with coarse-milling. As compared with conventional tofu, the employ of okara significantly decrease the springiness and cohesiveness of tofu without **MTG**ase treated. The decrease in cohesiveness resulted from the presence of okara causing less uniform and homogeneous microstructure. These observations were in agreement with our previous findings (*28*) that tofu with a less intensive network give less cohesiveness. Meanwhile, **MTG**ase treatment was expected to cause significantly increases in hardness, springiness, cohesiveness, gumminess and chewiness of tofu texture (Table 3). The much firmer texture was due to excessive formation of cross-linking (*21*), while subsequently found to decrease the acceptability of tofu by consumer. Besides, the coarse texture couldn't meet the requirement of consumers.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Table 1. Effect of particle size of okara on the moisture, WRA, protein recovery, solid recovery and tofu yield of “whole-soybean tofu”[a]

Tofu variety	*Soymilk Solid Content (%)*	*Moisture (%)*	***WRA*** *(%)*	*Protein Recovery (%)*	*Solid Recovery (%)*	*Tofu yield (g tofu/ 100 g soybean)*
Conventional tofu	6.0 ± 0.0 b	80.5 ± 0.5 c	77.2 ± 0.1 a	70.9 ± 0.7 b	55.9 ± 0.8 b	253.3 ± 2.9 c
Whole-soybean tofu [b]						
Coarse milling	8.0 ± 0.1 a	84.3 ± 0.8 b	52.4 ± 0.7 c	79.3 ± 0.9 a	78.8 ± 3.9 a	451.1 ± 1.5 b
Fine milling	8.0 ± 0.1 a	86.2 ± 0.4 a	55.3 ± 1.5 b	80.5 ± 1.0 a	76.1 ± 1.8 a	495.4 ± 6.5 a

[a] Mean scores bearing the same letters among the same column are not significantly different ($p < 0.05$). [b] The tofu made at the water-to-bean ratio of 10:1 and with 0.4% calcium sulfate in soymilk.

Table 2. Effect of transglutaminase on the moisture, WRA, protein recovery, solid recovery and tofu yield of “whole-soybean tofu” [a]

Tofu Variety	*Soymilk Solid Content (%)*	*Moisture (%)*	***WRA*** *(%)*	*Protein Recovery (%)*	*Solid Recovery (%)*	*Tofu Yield (g tofu/ 100 g soybean)*
Conventional tofu	6.0 ± 0.0 b	80.5 ± 0.5 b	77.2 ± 0.1 a	70.9 ± 0.7 c	55.9 ± 0.8 b	253.3 ± 2.9 b
Whole-soybean tofu [b]						
Without **mtg**ase	8.0 ± 0.1 a	84.3 ± 0.8 a	52.4 ± 0.7 c	79.3 ± 0.9 b	78.8 ± 3.9 a	451.1 ± 1.5 a
With **mtg**ase	8.0 ± 0.1 a	83.5 ± 0.8 a	70.4 ± 1.8 b	83.7 ± 1.0 a	83.2 ± 4.0 a	453.2 ± 3.9 a

[a] Mean scores bearing the same letters among the same column are not significantly different ($p < 0.05$). [b] This whole-soybean tofu was manufactured with coarse milling.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Table 3. Effect of transglutaminase on the textural properties of "whole-soybean tofu" [a]

Tofu Variety	*Hardness (Kg)*	*Springiness (mm)*	*Cohesiveness (mm)*	*Gumminess (Kg)*	*Chewiness (Kg.mm)*
Conventional tofu	0.71 ± 0.11 c	9.76 ± 0.04 b	0.45 ± 0.02 a	0.32 ± 0.02 b	3.11 ± 0.25 b
Whole-soybean tofu [b]					
Without **MTG**ase	1.06 ± 0.09 b	7.74 ± 0.33 c	0.27 ± 0.01 c	0.29 ± 0.03 b	2.22 ± 0.25 b
With **MTG**ase	3.68 ± 0.13 a	10.59 ± 0.18 a	0.38 ± 0.03 b	1.40 ± 0.12 a	14.81 ± 0.98 a

[a] Mean scores bearing the same letters among the same column are not significantly different ($p < 0.05$). [b] This whole-soybean tofu was manufactured through coarse milling.

Effects of Water-to-Bean Ratios on the Quality and Texture of "Whole-Soybean Tofu" Manufactured through Fine Milling

As mentioned earlier, the whole-soybean tofu manufactured through fine milling could form a stable and firm tofu gel just by means of calcium sulfate addition. As compared with coarse milling, this tofu gave better homogeneous appearance and smoother texture. Table 4 shows the tofu made with water-to-bean ratio of 12:1 had the maximal protein and solid recoveries, and the best **WRA** as well as the maximal tofu yield. As stated previously, the employ of okara resulted in increase in moisture content and subsequently caused decrease in tofu hardness (Table 5). In addition, okara might have a role to destroy the uniformity of tofu microstructure owing to its poor dispersion in soymilk, in turn, consequently, resulted in less cohesiveness. The results of Table 5 indicated that hardness, springiness, cohesiveness, gumminess, and chewiness decreased as the water-to-bean ratio increased. However, as compared with conventional tofu, the whole-soybean tofu treated with the water-to-bean-ratios in the range of 10-14, all possessed softer, less elastic and less chewy texture (Table 5).

Figure 1 shows the surface and cross-section photographs of conventional tofu and whole-soybean tofu manufactured via fine milling as we suggested above. The whole-soybean tofu was darker in color, had coarse surface and coarse cross-section, and rough mouthfeel as compared to conventional tofu. As stated earlier, Aburage and Kori- tofu were two tofu analogues, both with coarse appearance and rough mouthfeel but chewy texture. Therefore, we suggest this whole-soybean tofu could be served in following ways: cooked in soup, fried in oil (close to Aburage tofu), or further developed into Kori-tofu.

Table 4. Effect of water-to-bean ratios on the moisture, WRA, protein recovery, solid recovery and tofu yield of "whole-soybean tofu"[a,b]

Water:Bean Ratio	*Soymilk Solid Content (%)*	*Moisture (%)*	***WRA** (%)*	*Protein Recovery (%)*	*Solid Recovery (%)*	*Tofu Yield (g tofu/ 100 g soybean)*
10	8.0 ± 0.1 a	86.2 ± 0.4 a	55.3 ± 1.5 b	80.5 ± 1.0 c	76.1 ± 1.8 b	495.4 ± 6.5 b
12	7.2 ± 0.1b	85.7 ± 0.1a	60.9 ± 1.1 a	87.4 ± 0.5 a	80.7 ± 0.6 a	507.1 ± 6.1 a
14	6.0 ± 0.0 c	86.0 ± 0.3 a	59.9 ± 0.9 a	85.3 ± 0.5 b	78.0 ± 0.2 ab	501.1 ± 2.7 ab

[a] Mean scores bearing the same letters among the same column are not significantly different ($p < 0.05$). [b] This whole-soybean tofu was manufactured through fine milling.

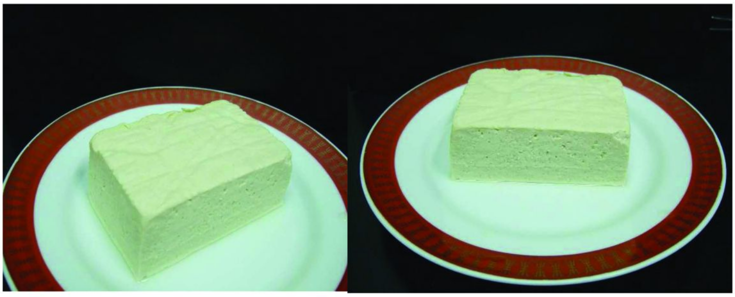
Table 5. Effect of water-to-bean ratios on the textural properties of "whole-soybean tofu" [a]

Water:Bean Ratio	*Hardness (kg)*	*Springiness (mm)*	*Cohesiveness (mm)*	*Gumminess (kg)*	*Chewiness (kg.mm)*
Conventional tofu	0.71 ± 0.11 a	9.76 ± 0.04 a	0.45 ± 0.02 a	0.32 ± 0.02 a	3.11 ± 0.25 a
Whole-soybean tofu [b]					
10	0.62 ± 0.04 b	6.97 ± 0.65 b	0.35 ± 0.02 b	0.22 ± 0.01 b	1.53 ± 0.02 b
12	0.59 ± 0.09 b	6.47 ± 0.40 bc	0.35 ± 0.02 b	0.21 ± 0.02 b	1.36 ± 0.11 b
14	0.55 ± 0.05 b	5.97 ± 0.25 c	0.30 ± 0.04 c	0.17 ± 0.01 c	0.99 ± 0.10 c

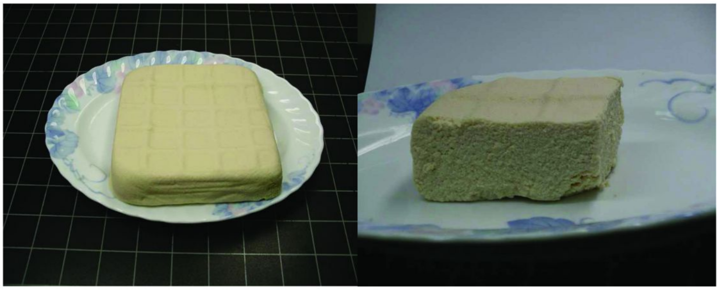
[a] Mean scores bearing the same letters among the same column are not significantly different ($p < 0.05$). [b] This whole-soybean tofu was manufactured through fine milling.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018



(A) Conventional tofu



(B) Whole-soybean tofu

Figure 1. Photograph of conventional tofu (A) and whole-soybean tofu manufactured via fine milling (B).

Sensory Evaluation Study

In this acceptance test of this new tofu, sample was served in cooked form, in the future study we would like to analyze the acceptance in the Aburage and Kori-tofu form. Tofu samples were evaluated by a panel for appearance, flavor, texture and overall acceptability on a 9-point hedonic scale. Conventional tofu and whole-soybean tofu were evaluated, and the results are shown in Table 6. With the employ of okara, there were trends of decrease acceptance for appearance, texture, and overall acceptance except for flavor. Appearance acceptance decreased in whole-soybean tofu for the sake of darker color and less smoother surface, while increase in flavor acceptance resulted from having a savory cooked flavor. A survey revealed that cooked okara could contribute pleasant cooked flavor to puffed okara/rice cake (*31*) supported our results. Regarding to texture acceptance, preference varies from place to place, in China and Taiwan there is a preference for a firmer and chewier mouthfeel (*20*, *32*, *33*). Therefore, the hardness and chewiness measured by texture analyzer were in agreement with our sensory evaluation result (Table 5**,** Table 6).

Table 6. Nine point scale sensory evaluation of conventional tofu and whole-soybean tofu [a]

Tofu Variety	*Appearance*	*Flavor*	*Texture*	*Overall Acceptability*
Conventional tofu	6.8 ± 1.3 a	6.3 ± 1.2 b	5.6 ± 1.4 a	6.5 ± 1.3 a
Whole-soybean tofu [b]	6.5 ± 1.4 b	6.5 ± 1.4 a	5.3 ± 1.2 b	6.2 ± 1.4 b

[a] Mean scores bearing the same letters among the same column are not significantly different ($p < 0.05$). [b] This whole-soybean tofu was made at the water-to-bean ratio of 12:1 and fine milling.

Table 7. The general compositions of soybeans, tofu, okara and whole-soybean tofu

Sample	*Moisture (%)*	*Solids*				
		protein (%)	*lipid (%)*	*ash (%)*	*carbo-hydrate (%)*	*crude fiber (%)*
raw soybean	10.1 ± 0.5	40.6 ± 2.1	22.0 ± 1.9	6.1 ± 0.5	23.7 ± 0.3	7.6 ± 2.3
conventional tofu	80.5 ± 2.5	50.2 ± 0.5	34.1 ± 2.1	7.2 ± 0.3	8.5 ± 0.4	0.0 ± 0.0
okara	79.2 ± 3.1	22.5 ± 2.1	8.0 ± 2.1	5.0 ± 0.4	45.2 ± 4.1	19.3 ± 0.3
whole-soybean tofu[a]	86.3 ± 2.6	46.4 ± 3.1	23.2 ± 1.8	6.4 ± 0.2	14.9 ± 2.0	9.1 ± 0.6

[a] This whole-soybean tofu was made at the water-to-bean ratio of 12:1 and fine milling.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Food Chemical Analysis of Soybean, Okara, Tofu, and Whole-Soybean Tofu

Results of the food chemical analyses are presented in Table 7. The conversion of soybeans into tofu and okara led to the following changes in the proximate composition, protein and lipid were concentrated in tofu, while carbohydrate and crude fiber were almost concentrate in okara. Regarding the whole-soybean tofu, as compared with conventional tofu, the employ of okara was found to result in the increase in carbohydrate and crude fiber content. While, as compared with raw soybean, there was a decrease in carbohydrate content that might be due to the loss of soluble carbohydrate, including monosaccharides and oligosaccharides, during tofu-manufacturing. Besides, the poor **WRA** might be the reason to cause the loss of soluble carbohydrate.

In conclusion, okara is the byproduct during soymilk or tofu manufacturing. It's high in nutrients and possesses great potential to be applied to functional human food. The object of this study was to develop whole-soybean tofu or its analogue

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

and could commercialize it in the future, thus, it would improve human health and decrease the risk levels of ecological damage. In this study, we found that whole-soybean tofu with fine-milling could form a stable firm gel just after addition of calcium sulfate, while tofu with coarse milling couldn't be successful made until by means of **MTG**ase addition. On the other hand, **MTG**ase treatment was expected to increase significantly hardness, springiness, cohesiveness, gumminess and chewiness of tofu texture, as well as **WRA**.

Regarding to the whole-soybean tofu with fine milling, it was found that tofu made with water-to-bean ratio of 12:1 gave the maximal protein and solid recoveries, as well as the maximal tofu yield. However, this whole-soybean tofu possessed softer, less chewy texture, and coarse appearance. Therefore, **MTG**ase will be still needed to modify tofu texture in the following research. This whole-soybean tofu could be served in the following ways: cooked in soup, fried in oil, or further developed into Kori-tofu. Above all, whole-soybean tofu was rich in fiber and low in fat, it could be considered as a healthy food.

Acknowledgments

The authors thank the National Science Council of the Republic of Chins, Taipei, Taiwan, for the financial support of this research under the project NSC-89-2214-E-002-076 and NSC-90-2214-E-002-028.

References

1. Liu, K. S.; Orthoefer, F.; Brown, E. A. Association of seed size with genotypic variation in the chemical constituents of soybeans. *J. Am. Oil Chem. Soc.* **1995**, *72*, 191–196.
2. Wang, H.; Murphy, P. A. Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem.* **1994**, *42*, 1674–1677.
3. Saio, K. Tofu-relationships between texture and fine structure. *Cereal Foods World* **1979**, *24*, 342–354.
4. Wang, H. L.; Hesseltine, C. W. Coagulation conditions in tofu processing. *Process Biochem.* **1982**, *1*, 7–12.
5. Wang, H.; Swain, E. W.; Kwolek, W. F. Effect of soybean varieties on the yield and quality of tofu. *Cereal Chem.* **1983**, *60*, 245–248.
6. Bourne, M. C.; Clemente, M. G.; Banzon, J. Survey of the suitability of thirty cultivars of soybeans for soymilk manufacture. *J. Food Sci.* **1976**, *41*, 1204–1208.
7. O'Toole, D. K. Characteristics and use of okara, the soybean residue from soy milk production. A review. *J. Agric. Food Chem.* **1999**, *47*, 363–371.
8. Jackson, C. J. C.; Dini, J. P.; Lavandier, C.; Rupasinghe, H. P. V.; Faulkner, H.; Poysa, V.; Buzzell, D.; DeGrandis, S. Effects of processing on the content and composition of isoflavones during manufacturing of soy beverage and tofu. *Process Biochem.* **2002**, *37*, 1117–1123.

9. Anderson, J. W.; Johnstone, B. M.; Cook-Newell, M. E. Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* **1995**, *333*, 276–282.
10. Yoshiki, Y.; Kudou, S.; Okubo, K. Relationship between chemical structure and biological activities of triterpenoid saponin from soybean. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 2291–2299.
11. Chan, W. M.; Ma, C. Y. Acid modification of proteins from soymilk residue (okara). *Food Res. Int.* **1999**, *32*, 119–127.
12. Yoshii, H.; Furuta, T.; Maeda, H.; Mori, H. Hydrolysis kinetics of okara and characterization of its water-soluble polysaccharides. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1406–1409.
13. Ohno, A.; Ano, T.; Shoda, M. Use of soybean curd residue, okara, for the solid state substrate in the production of a lipopeptide antibiotic, Iturin A, by *Bacillus subtilis* NB22. *Process Biochem.* **1996**, *31*, 801–806.
14. Zhu, Y. P.; Fan, J. F.; Cheng, Y. Q.; Li, L. T. Improvement of the antioxidant activity of Chinese traditional fermented okara (Meitauza) using *Bacillus subtilis* B2. *Food Control* **2008**, *19*, 654–661.
15. Kurokochi, K.; Matsuhashi, T.; Nakuzawa, M.; Nakazawa, A. Use of okara powder for bread. *New Food Ind.* **1977**, *19*, 49–53.
16. Khare, S. K.; Jha, K.; Sinha, L. K. Preparation and nutritional evaluation of okara fortified biscuits. *J. Dairy., Foods Home Sci.* **1995**, *14*, 91–94.
17. Genta, H. D.; Genta, M. L.; Alvarez, N. V.; Santana, M. S. Production and acceptance of a soy candy. *J. Food Eng.* **2002**, *53*, 199–202.
18. Ku, K. H.; Kim, M. J.; Kim, N. Y.; Chun, H. S. Effects of microparticulated soybean powder and its preparation condition on textural properties of Chundubu. *Food Sci. Biotechnol.* **2001**, *10*, 211–218.
19. Ku, K. H.; Kim, M. J.; Lee, M. K. Effects of enzyme treatment on the physical properties of microparticulated soybean powder. *Food Sci. Biotechnol.* **2002**, *11*, 380–388.
20. Cai, T. D.; Chang, K. C. Dry tofu characteristics affected by soymilk solid content and coagulation time. *J. Food Qual.* **1997**, *20*, 391–401.
21. Yasir, S. Bin. Md.; Sutton, K. H.; Newberry, P.; Andrews, N. R.; Gerrard, J. A. The impact of transglutaminase on soy proteins and tofu texture. *Food Chem.* **2007**, *104*, 1491–1501.
22. *Official Methods of Analysis*, 13 ed.; Association of Official Analytical Chemists: Washington, DC, 1980.
23. Puppo, M. C.; Añón, M. C. Structural properties of heated-induced soy protein gels as affected by ionic strength and pH. *J. Agric. Food Chem.* **1998**, *46*, 3583–3589.
24. Bourne, M. C. Texture profile analysis. *Food Technol.* **1978**, *32*, 62–66.
25. *SAS/STAT User's Guide*, version 6.03; SAS Institute, Inc.: Cary, NC, 1990.
26. Ma, C. Y.; Liu, W. S.; Kwok, K. C.; Kwok, F. Isolation and characterization of proteins from soymilk residue (okara). *Food Res. Int.* **1996**, *29*, 799–805.
27. Toda, K.; Chiba, K.; Ono, T. Effect of components extracted from okara on the physicochemical properties of soymilk and tofu texture. *J. Food Sci.* **2007**, *72*, C108–C113.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

28. Kao, F. J.; Su, N. W.; Lee, M. H. Effect of calcium sulfate concentration in soymilk on the microstructure of firm tofu and the protein constitutions in tofu whey. *J. Agric. Food Chem.* **2003**, *51*, 6211–6216.
29. Nonaka, M.; Sakamoto, H.; Toiguchi, S.; Yamagiwa, K.; Soeda, T.; Motoki, M. Retort resistant tofu prepared by incubation with microbial transglutaminase. *Food Hydrocolloids* **1996**, *10*, 41–44.
30. Kwan, S. W.; Easa, A. M. Comparing physical properties of retort-resistant glucono-d-lactone tofu treated with commercial transglutaminase enzyme or low levels of glucose. *Lebensm.-Wiss. Technol.* **2003**, *36*, 643–646.
31. Xie, M.; Huff, H.; Hsieh, F.; Mustapha, A. Puffing of okara/rice blends using a rice cake machine. *J. Food Sci.* **2008**, *73*, E341–E348.
32. Tsai, S. T.; Lar, C. Y.; Kaq, L. S.; Cher, S. C. Studies on the field and quality characteristics of tofu. *J. Food Sci.* **1981**, *46*, 1734–1737, 1740.
33. Wang, H.; Swain, E. W.; Kwolek, W. F. Effect of soybean varieties on the yield and quality of tofu. *Cereal Chem.* **1983**, *60*, 245–248.

Downloaded by UNIV OF GUELPH LIBRARY on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch018

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Chapter 19

Soy Protein Functionality and Food Bar Texture

Myong J. Cho[*]

Solae, LLC, St. Louis, MO 63110
[*]mcho@solae.com

Six Sigma Methodology was employed to investigate the relationship between the chemical and physical properties of isolated soy proteins (ISP) and the food bar texture based on mechanical hardness and sensory during accelerated storage. Selected bar model systems with high protein content at ≥ 30% were used in the study. The mechanical bar hardness measured by Texture Analyzer was found to be correlated with the hedonic Overall Liking score in sensory evaluation of the high protein bars. Five critical properties of the soy protein important to the bar texture were identified. They are protein solubility, degree and type of enzymatic protein hydrolysis, density, particle size, and particle surface morphology. The protein solubility and degree of hydrolysis are the primary protein properties most significantly affecting the bar hardness and sensory. Density is a secondary property revealed after the primary properties are controlled. Effect of particle size is shown when the first and secondary properties are comparable within narrow ranges. Commercially available ISP's, Supro® 320, Supro® 313 and Supro® 430 developed from this study can provide technical solutions for creating desirable texture and shelf life in the high protein food bar formulations containing various dairy proteins and sugar syrup levels.

Introduction

Soy protein has been widely used in a variety of food applications from emulsified meat to acidic and neutral beverages and infant formulas. The isolated soy protein (ISP) containing at least 90% protein on dry weight basis is an excellent alternative to dairy proteins such as caseinate, milk protein isolate and

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

whey protein isolate. The ISP provides all required essential amino acids set by FAO/WHO (*1*). In addition, soy protein has been the major source of protein in beverage applications for a population suffering from lactose intolerance as well as it has been successfully used in meat analog products for vegetarians. Furthermore, in 1999 the Food and Drug Administration (FDA) of U.S.A. has recognized soy protein as useful for lowering blood cholesterol level by stating that "25 grams of soy protein a day as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease (*2*)." Since then, soy protein has been utilized increasingly as a preferred protein source in various healthy foods.

Food bars are defined as combinations of ingredients that provide food in a solid, low-moisture form (*3*). The food bars are consumed as a source of nutrients and therefore they are formulated to contain a wide range nutrient and positioned in various ways for consumers. A first-generation high protein bar was developed by Nebisco Brands, Inc. in 1985 to provide a low lactose feature with all necessary vitamins and minerals. This high protein bar was targeted to have a shelf life of at least 6 to 12 months (*4*). However, food bars have become a popular consumer choice since 2000. One primary reason for their popularity is that as defined food bars are frequently used as a nutrient source for people who do not have time for a meal. Another reason is that high protein food bars are used by athletes to enhance athletic performance and help build body mass. Confectionery food bars have been used in weight loss programs by the health conscious consumers as low-calorie "meal replacers". More recently, the high protein low carbohydrate food bars have become popular to diabetic consumers. The high protein low carbohydtrate bars showed a significantly lower glycemic index than the low protein high carbohydrate bars (*5*). As a result, the food bar industry has grown tremendously in the past decade, showing $3 billions in 2005 and expecting to reach $5 billions by 2010. The food bars are normally formulated to contain proteins, carbohydrates, and flavorings. The protein and carbohydrate levels are depending on the types of bar formulations. The serving size in the US market varies from 28 to 80 grams with a typical range at 50 to 56 grams (*3*). The protein contents vary from 6-8 grams/serving for meal replacement type bars to 35% protein/serving or higher for high protein bars. The carbohydrate levels vary from 35-38 grams/serving for the meal replacement bars to 2-6 grams/serving for the high protein bars (*3*). Most high-protein bar formulations employ dairy proteins such as calcium caseinate, whey protein concentrate and whey protein isolate.

The food bars must include relatively high levels of soy protein in the formulation to promote soy health benefits. Inclusion of high levels of protein in a food bar, however, negatively affects texture, palatability and shelf life of the food bar, relative to food bars containing less protein and more carbohydrates. The high protein bar formulation containing more than 20% protein tends to be hard during shelf life. The mechanism for hardening has not been well understood because of the complexity in formulations of ingredients and their sources along with bar processing conditions (Figure 1).

One theory for the bar hardening during storage is water migration from one ingredient to another in the bar formulation. This water migration likely brings changes in the bar structure and hardening phenomena primarily caused by those ingredients losing moisture to others over time. Normally, the water activity

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

tends to increase during the accelerated storage, but has no correlation with bar hardness. On the other hand, type of protein and carbohydrate significantly affect the bar hardness. Li, et al. used NMR relaxometry for studying the spin-spin relaxation times of various powdered proteins and syrups over a temperature range to determine any correlation between proteins and bar hardiness (*6*). Increase in relaxation times of the proteins seems to be related to better performance in the bars. The relaxation time is affected by chemical and physical surrounding of the spins and it appears to be related to the protein properties. Currently, little literature information is available regarding a correlation between protein functionality and bar hardening phenomena. Furthermore, there is no published research specifically on soy protein functionality related to food bar texture.

The objective of this research was to identity the functionality of soy proteins critical to texture of food bars containing 30% or higher protein and develop soy protein ingredients for improving the bar texture as well as extending bar shelf life with acceptable textural properties.

Materials and Methods

Experimental Approach

Six Sigma methodology (*7*) was employed to identify various functional attributes of isolated soy protein (ISP) and their relationships to food bar texture. There are five phases in the Six Sigma method: 1) Define Phase for collecting voice of customers (VOC) to determine prevailing defects and identifying those parameters critical to quality (CTQ) to fix the respective defects. The top CTQ for this study was to identify critical ISP properties to food bar texture. 2) Measure Phase to identify and standardize the measurement systems to generate bar texture data ("Project Y").

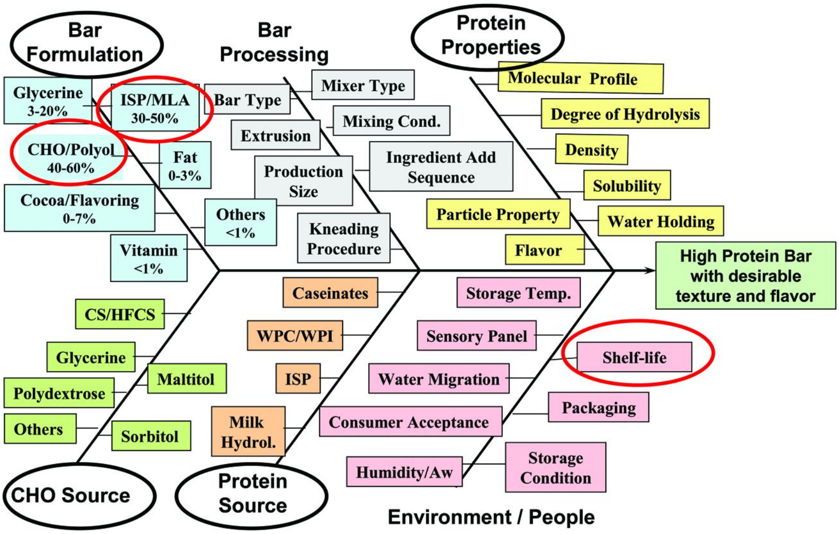

Bar Formulation
Bar Processing
Protein Properties
Glycerine 3-20%
ISP/MLA 30-50%
Bar Type
Mixer Type
Mixing Cond.
Extrusion
Molecular Profile
Degree of Hydrolysis
CHO/Polyol 40-60%
Fat 0-3%
Production Size
Ingredient Add Sequence
Density
Solubility
Cocoa/Flavoring 0-7%
Others <1%
Kneading Procedure
Particle Property
Water Holding
Vitamin <1%
Flavor
High Protein Bar with desirable texture and flavor
Caseinates
Storage Temp.
CS/HFCS
WPC/WPI
Sensory Panel
Shelf-life
Glycerine
Maltitol
ISP
Water Migration
Polydextrose
Packaging
Others
Sorbitol
Milk Hydrol.
Consumer Acceptance
Humidity/Aw
Storage Condition
CHO Source
Protein Source
Environment / People

Figure 1. Cause – Effect Diagram for High Protein Bar System (see color insert)

The measurement systems in this study included the mechanical hardness measured by Texture Analyzer for all bar samples, and an informal sensory for selected food bar formulations in early stage. 3) Analyze Phase to analyze various experimental samples in respect to the current defect protein, correlate various ISP properties that were hypothesized and identify a few critical ISP properties ("vital few X") for improving the bar texture. These vital few Xs may lead to draw a hypothesis for improving the bar texture based on the improvement goal, i.e. "reduced bar hardness than the existing ISP in the market." 4) Improve Phase to pilot the solution based on the hypothesis by modifying the vital few Xs. 5) Control Phase to produce the target ISP under the process condition yielding the modified vital few Xs. Figure 1 illustrates a Cause-Effect diagram used in the Six Sigma experimental approach demonstrating the extent of complexity associated with the high protein food bar system.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Soy Protein Material

Experimental and Commercial ISP Products

The various experimental ISP samples used in the Six Sigma experiments for developing correlation data in this study were produced according to the base process covered under US Patent 7,419,695 B1 (*8*). Supro® 320, Supro® 313 and Supro® 430 manufactured by Solae, LLC (St. Louis, MO) are the commercial ISP products developed from the Six Sigma Project and their processes are also covered in US Patent 7,419,695 B1. Supro® 661 also manufactured by Solae, LLC (St. Louis, MO) was used as the baseline reference for the improved proteins.

Ground Material of Supro® Nuggets 311 for Particle Size Study

Two ground soy protein materials were prepared to demonstrate the particle size effect on bar hardness without varying other physical and chemical properties. Supro® Nuggets 311 (Solae, LLC, St. Louis, MO) was ground to two different particle size ranges at a commercial grinding facility: One was "Coarse Ground" with >20% on 100 mesh (150 μm) and the other "Fine Ground" with 0% on 100 mesh. Supro® Nuggets 311 containing 80% soy protein has a particle size target of 80% on 6 mesh (3.35 mm).

Blends of Supro® 320 and Supro® 313 for Bar Application Test

A series of blends at 10:90 to 90:10 of Supro® 320 and Supro® 313 were prepared for testing in the 30% protein all soy and all sugar syrup bar formulation.

Food Bar Formulations, Production and Accelerated Storage

Two food bar model formulations were used in this study. One was an all soy, all sugar syrup formulation used throughout the Six Sigma experiments and for testing the commercial ISP products, and the other was a high protein, low carbohydrate formulation used for testing the new single protein solution, Supro® 430.

All Soy, All Sugar Syrup Bar Formulation

The food bar production procedure based on the formulation (Table 1) has three stages. The base procedure for the bar preparation is covered under US Patent 7,419,695 B1 (*8*). The dry ingredients were mixed in a Winkworth mixer (Winkworth Machinery, Ltd., Reading, England) for 1 minute at 48 rpm. The pre-heated syrup at 60°C containing the flavors/glycerin was added to the dry ingredient blend and mixed at 50°C for 3 minutes 45 seconds to form dough. The dough was sheeted onto a marble slab and cut to form a food bar. A number of food bars required for initial and storage studies were made for each of the respective protein formulations. Whole bar length was approximately 100 mm long x 36 mm wide. The bars were packaged individually in oxygen-barrier pouches (Silver Paks, Ampac, Cincinnati, OH) and heat-sealed.

High Protein, Low Carbohydrate Formulation (35-40% Protein)

Detail formulations are described in US Patent Application Publication 2007/0042103 A1 (*9*). A first mixture was produced in a Winkworth mixer (Winkworth Machinery, Ltd., Reading, England) mixing at a speed of 48 rpm for one minute. The first mixture comprised 933.4 grams protein material (33% protein from ISP), 116.0 grams cocoa powder (DeZaan, Milwaukee, WI), 14.0 grams vitamin and mineral premix (Fortitech, Schenectady, NY), 2.0 grams salt, 0.6 grams sucralose (Splenda® from Tate & Lyle, Inc., Decatur, IL), 144.0 grams shortening (BakeMark, Bradley, IL), and 14.0 grams lecithin (Centrophase 152 from Solae LLC, St. Louis, MO). In a separate container, a second mixture containing carbohydrate material and liquid flavoring agents was heated to a temperature of 100°F (37.8°C) by microwaving the mixture on high power for about 45 seconds. The carbohydrate material consisted of a mixture of 59.1 grams glycerin, 94.0 grams maltitol, 94.0 grams polydextrose syrup. The liquid flavoring agents consist of a mixture of 8.0 grams Edlong Chocolate flavor 610 (The Edlong Corporation, Elk Grove Village, IL), 8.0 grams Edlong Chocolate flavor 614 (The Edlong Corporation, Elk Grove Village, IL), and 6.0 grams vanilla flavoring (available from Sethness Greenleaf, Inc., Chicago, IL). The first mixture was combined with the second mixture in the Winkworth mixer and mixed at a speed of 48 rpm for three minutes and forty-five seconds. The resulting dough was sheeted onto a marble slab and bars are cut into pieces weighing from about 45 grams to about 55 grams (the bar pieces are 10.5 mm in length, 10 mm in height, and 4.5 mm wide). The bars were packaged individually oxygen-barrier pouches (Silver Paks, Ampac, Cincinnati, OH) and heat-sealed.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Table 1. Formulation of All Soy, All Sugar Syrup Bar with 30% Protein

Ingredients	*% Total*
Stage 1 (Dry Ingredients)	
Powdered ISP	34.2
Rice syrup solids, 26 DE	7.88
Cocoa Powder	5.07
Vitamin and Mineral premix	0.70
Salt	0.11
Stage 2	
Corn Syrup, 63 DE	26.1
HFCS	21.4
Stage 3	
Glycerin	3.95
Chocolate Flavor Elong # 614	0.27
Chocolate Flavor Elong # 610	0.27
Vanilla Flavor	0.13
Total	**100.0**

Rice Syrup Solids 26DE: California Natural Products (Lathrop, CA), lot# 405453-01348
Cocoa powder: DeZaan (Milwaukee, WI), 11-D-188, lot# 305111022
Vitamin and Mineral Premix FT961279: Fortitech (Schenectady, NY), lot# 0203041
Salt: Cargill Foods, lot# HO232
63 DE corn syrup: International Food Ingredients, lot# 02051
55 HFCS: International Food Ingredients, lot# A02122 D-4
Glycerine (99.7%): Proctor and Gamble, CAS# 56-81-5
Chocolate flavor: Edlong (Elk Grove, IL), lot# 6007, F005601, lot# 757468
Dark Chocolate flavor (Nat): Edlong, lot# 6147; F005602, lot# 757471
Vanilla flavor 4x: Sethness Greenleaf (Chicago, IL), lot# 1721

Accelerated Storage Study

All food bar samples (except for Day 1 fresh bars) were stored at 32°C (90°F) in a controlled environment chamber up to 42 days. The bars were removed after 7, 14, 21, 35 and 42 days of storage for texture measurement. The food bars were equilibrated to room temperature before the test.

Analytical Methods

Protein Solubility

The solubility of the protein samples was measured as 'Soluble Solids Index (SSI)' described in US Patent 7,419,695 B1 (*7*). A 2.5% slurry was prepared by dispersing protein powder in water with a blender at room temperature, mixed

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

for 30 minutes and then centrifuged at 500xg for 10 minutes. The solid contents of total (before centrifugation) and its supernatant (after the centrifugation) were determined. SSI was calculated as follow:

$$\text{SSI (\%)} = (\text{Soluble Solids}/\text{Total Solids}) \times 100$$

Degree of Hydrolysis

The "trinitrobenzene sulfonic acid" test ("TNBS") was used to measure of the degree of hydrolysis (DH) of soy proteins. The detailed procedure is described in US Patent 7,419,695 B1 (*8*). This is a simplified TNBS procedure (STNBS) originated from Fields (*10*). Primary amines occur in soy proteins as amino terminal groups and also as the amino group of lysyl residues. The process of hydrolysis cleaves the peptide chain structure of soy proteins creating one new amino terminal with each break in the chain. The intensity of color developed from a TNBS-amine reaction is proportional to the total number of amino terminal groups in a soy protein sample, and, therefore, it is an indicator of the degree of hydrolysis of the protein in the sample. The STNBS value (moles $NH_2/10^5$ g protein) is defined according to the following formula:

$$\text{STNBS} = (As_{420} - Ab_{420}) \times 8.073 \times 10 \times F \times 100/P$$

where As_{420} is the absorbance of a TNBS sample solution at 420 nm; Ab_{420} is the absorbance of a TNBS reagent blank at 420 nm; 8.073 is a calculated value of extinction coefficient and dilution/unit conversion factor in the procedure; F is the dilution factor; and P is the protein content of the sample by Kjeldahl or Leco Combustion method.

Size Exclusion Chromatography (SEC) Molecular Weight Profile

Selected samples were analyzed for molecular weight distribution (MWD) using an Agilent 1100 High Pressure Liquid Chromatography (HPLC) system. The HPLC system employed a ZORBAX GF-250 (9.4 x 250 mm) column from Agilent Technologies (Santa Clara, CA). This HPLC system was equipped with a UV detector, an autosampler and a HPLC software program from Agilent ChemStation. The MW calculation was done based on a correlation equation between molecular weight standards and elution time. The mobile phase was 6M guanidine hydrochloride (GuHCl) with dithiothreitol (DTT) in 0.1 M phosphate buffer. This mobile phase was designed for dissociating protein completely to its subunit. MW standard proteins (all from Sigma Aldrich Chemicals, St. Louis, MO) used for calibrating the columns were: hexapeptides (686), Vitamin B12 (1,355), aprotinin (6,500), cytochrome C (12,400), myoglobulin (17,000), α-chymotrypsin (25,700), ovalbumin (44,000) and bovine serum albumin (BSA, 66,000 daltons). Designated amounts of the protein standards and protein samples at 0.5% were completely dispersed in mobile phase, centrifuged at 31,300 x g for 20 minutes to remove the insoluble fraction, and filtered through 0.45 micron cellulose acetate membrane. The filtered samples were transferred to the autosampler for the analysis. The UV absorbance was monitored at 280 and 260 nm.

Polyacrylamide Gel Electrophoreses (PAGE)

One part of 1% sample solution is dispersed in 1 part of the Laemmli sample buffer (*11*) which is a Tris-HCl buffer (Bio-Rad, Cat. # 161-0731) containing 2% Sodium Dodecyl Sulphate (SDS) and with 5% mercaptoethanol. A precast 10-20% polyacrylamide Tris-HCl gradient gel is then applied to separate proteins. Bromophenol blue is used to mark the front boundary of the sample. The protein bands are stained with Coomassie Blue (Bio-Safe Coomassie Stain Cat# 161-0787). Gels can be dried and scanned to provide a record of the profile. Detailed procedures should be referred to Criterion Gels Application Guide (Bio-Rad 161-0993), Bio-Rad Quantity One Software Instruction Manual (Chapter 7, Appendix D), and Bio-Rad Gel Air Drying Frame Instructions. SDS-PAGE separates proteins by molecular size. SDS is used to eliminate charge differences between proteins which can affect electrophoretic separation (*12*). The polyacrylamide gel provides a sieving media for the electrophoretic separation. The gel is formed by cross-linking acrylamide with bis-acrylamide. The pore size is determined by the acrylamide concentration and the amount of cross-linking. Proteins moving through the gel which is related to the pore size of the gel and the radius of the protein molecules.

Particle Size Measurement

The particle size of protein powder was measured with Mastersizer 2000 with Scirocco 2000 dry powder feeder (Malvern Instruments Ltd., Worcestershire, UK). The detailed procedures should be referred to the user's manuals of Mastersizer 2000 and Scirocco 2000.

Density

A bulk density was measured by weighing a unit volume of powder in a flat top graduated cylinder designed for density. Density = Weight (g)/Volume (cc)

Scanning Electron Microscopy

The protein powder samples were submitted to Materials Evaluation and Engineering, Inc. (Plymouth, MN) for micrographs by scanning electron microscopy (SEM) characterization. SEM was done at 200, 500 and 1000x magnifications.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Mechanical Hardness of Food Bar Formulations

The mechanical hardness of the food bars was measured according to the procedure described in US Patent 7,419,695 B1 (*8*). The mechanical hardness was expressed in the grams of force necessary to compress the bar a preset distance using a probe connected to Texture Analyzer (TA), Model TA. XT2i (Texture Expert, Scarsdale, NY) with a 25 kg load cell and TA-55 probe, and Texture Expert Exceed Software. The probe was calibrated by setting the distance of the probe as close as possible to the Analyzer platform. The Texture Analyzer was set to move the probe 1 mm/sec. at a force of 100g, and the probe was driven into the food bar up to half the height of the food bar. The Texture Analyzer was also set to acquire 200 data points per second during the insertion of the probe into the food bar. The hardness of the food bar was measured in six replicates using two bars with three measurements (center and both ends) and calculated the average.

Bar Dough Stickiness

Bar dough stickiness was determined using a fixed amount of dough (before making bars) based on its adhesiveness (negative peak area) measured by Texture Analyzer.

Informal Sensory of Food Bar for Chewiness

An informal sensory panel of 5-10 panelists was used to determine the sensory chewiness of food bars in the early stage of this study. Each panelist independently rated the chewiness of each bar sample based on a 0 (not chewy) to 5 (very chewy) for breaking the bar matrix down. Samples were randomized and presented in duplicate.

Formal Sensory Acceptance of Food Bars for Overall Liking

A consumer panel consisted of 50-80 Nestle Purina Pet Care Company (St. Louis, MO) and Solae, LLC (St. Louis, MO) employees willing to evaluate chocolate flavored high protein food bars was employed as judges each time. A nine-point Hedonic Acceptance scale (1 = Dislike Extremely; 9 = Like Extremely) was used for determining Overall Liking along with other Liking sensory attributes such as appearance, flavor, texture and firmness. Bars were unwrapped, cut into three pieces with ends removed, and served on 6″ coded white plates. The samples were presented to panelists one at a time using a Williams 5 x 4 balanced incomplete block serving design, in which panelists evaluated 4 bars. Data analysis was done by Analysis of Variance for panelist and sample effects with mean separations using Tukey's Honestly Significant Difference (HSD) Test on Least Squares (Adjusted) Means. The Overall Liking data collected over a period of time for various experimental bar samples were used to generate a statistical correlation between the sensory and the bar hardness data from Texture Analyzer.

Results and Discussion

The Cause-Effect diagram (Figure 1) demonstrates many factors affecting the texture and flavor of the high protein food bars. Among these, protein and carbohydrate (or polyol) sources, and their properties are most critical to the bar texture, particularly for a high protein (>35% protein) bar formulation. The initial Six Sigma study used a fixed processing condition and a bar formulation (Table 1) containing soy as the sole source of protein and sugar syrup as the carbohydrate source to investigate the relationship between soy protein properties and bar texture primarily measured by the mechanical hardness based on Texture Analyzer (TA). The TA hardness is measured as the peak force during the first compression cycle.

The mechanical hardness (force in gram) measured by TA was found to be correlated with the formal sensory Overall Liking scores based on 28 sensory panels consisting of 1855 panelist responses (Figure 2, $R^2 = 0.82$). Therefore, the mechanical hardness was the focal measurement for bar texture in this study. It predicts that a bar at 2000 grams of force would have an overall liking score of 4.7. Bars are considered unacceptable once Overall Liking drops below 5.0.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

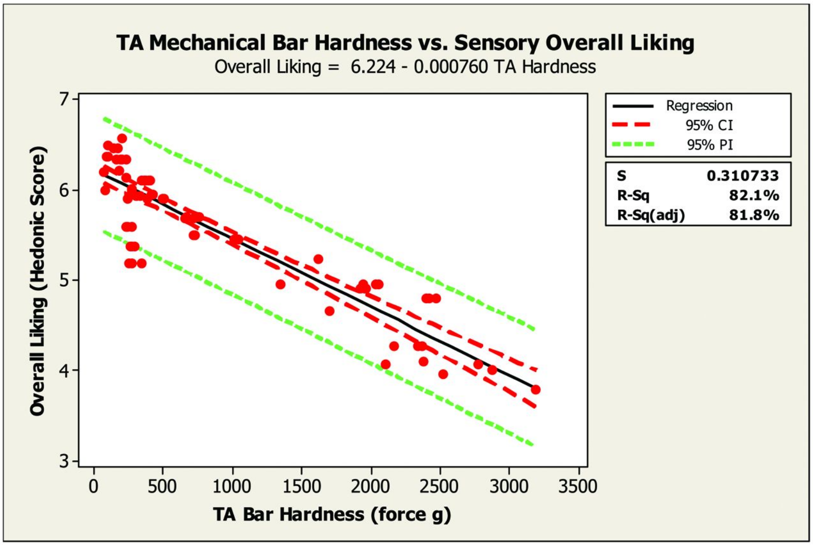


Figure 2. Mechanical Bar Hardness vs. Sensory Overall Liking

The texture of the initial food bar as well as its shelf life is significantly affected by protein properties such as solubility, degree and type of hydrolysis, particle size, density and particle surface morphology. The bar hardness increased during accelerated storage. It is difficult to see a high correlation between any single property and bar hardness when various products with different physical and chemical properties are compared across different functional proteins. However, based on the data analysis by the Six Sigma methodology, solubility turned out to be the most dominating factor followed by degree of hydrolysis. Particle size and density effects surfaced after these two primary factors were controlled to the optimum ranges.

There is no clear understanding on how each protein property is affecting the initial bar dough texture and bar hardening during shelf life. Based on the NMR studies by Li, et al. (*6*), the bar hardening during storage appears to be related to water migration pattern controlled by the types of protein and carbohydrate. The water migration naturally occurs when ingredients with different moisture contents contact each other. Water typically migrates from higher water activity liquid carbohydrate source (sugar syrup or sugar alcohol) to lower water activity powder proteins, since there is no other water source in the formulation. Physical and chemical properties of the protein powders that affect the extent and speed of water migration more likely influence the degree and rate of the bar hardening during storage. Protein competes with other ingredients for limited amount of water in the bar formulation. Soy protein with high solubility, particularly the intact protein with high water holding capacity, would require more water in order to be mixed in the bar formulation than those with low but optimum solubility. This would result in the other ingredients being deprived of water in the system, yielding a bar with more crumbly texture. There can be three types of water in food bars: bound water, free water and intermediate water. It is believed that the intermediate water acting as a plasticizer has the greatest effect on softness of bars. Lin, et al. (*13*) studied water migration using NMR, measuring mobility of the proton-containing compounds such as water, proteins, and carbohydrates. Spin-spin relaxation time (T_2) is used as an indication of proton mobility. In the high protein food bars, higher T_2 may be associated with a softer bar texture, but it may also indicate that the bar is more subject to quality change. Li et al. (*6*) used NMR relaxation times to categorize proteins based on the shapes of their individual NMR state diagram curves.

Soy Protein Functionality and Bar Hardness

Effect of Protein Solubility

Protein solubility of soy protein depends on its processing conditions such as process solids, temperature, pH, and spray-drying conditions. When the protein is treated with enzyme, the solubility is affected by the extent of hydrolysis. The protein solubility can be measured by two methods: one is based on Soluble Solids Index (SSI) described above, and the other is based on Nitrogen Solubility Index (NSI). In ISP, SSI closely matches NSI value.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

The protein solubility showed a high correlation with the bar hardness (Figure 3, $R^2 = 0.86$). The bar hardness increased as the solubility increased above 55% or decreased below 30% SSI. The optimum solubility for the softest texture was at around 40% SSI. The left side of the curve in Figure 3 was actually confirmed by the experimental products with 15-25% SSI producing hard bars. The proteins with higher or lower solubility than the optimum range tended to produce poor bar integrity. These proteins produced very dry and crumbly dough which were difficult to be molded into bars. The proteins with very low solubility could not have enough soluble protein and cohesive nature to pull other ingredients together in the bar processing, resulting in crumbly dough. On the other hand, the proteins with high solubility pull more water out of the system causing other ingredients to lack moisture. For example, sugar syrup could be gradually crystallized as it loses moisture to protein powder requiring more water. This would also result in crumbly dough.

A significant correlation between the bar hardness and solubility within the enzyme-treated soy proteins is demonstrated in Figure 4. The bar hardness increased as the solubility increased. However, it was observed that the enzyme-treated proteins with high solubility behave differently from the intact proteins with no enzyme treatment. They produced very soft and sticky dough making relatively soft bars initially, and then the bars became very chewy and hard over time, particularly in the sugar syrup bar formulation. This could be related to different water migration pattern changed over time from those of the intact proteins. The mechanism how water migration pattern changes over time depending on the protein and carbohydrate ingredient types is not well understood. It appears to be much more complex than involving water holding capacity (WHC) of protein and water activity within the bar formulation. Typically, soy protein has higher WHC than dairy protein, and the intact soy protein has higher WHC than the enzyme-treated soy protein. The enzyme-treated protein does not uptake much water and allows more water to be available in the bar matrix. This intermediate water could act as a plasticizer yielding a soft texture. The available amount of the intermediate water appears to depend primarily on protein types. The intermediate water would become free over time and no longer function as a plasticizer, resulting in bar hardening. The NMR study by Lin, et al also indicates that with accelerated storage more structure water is becoming free water (*13*).

Effect of Degree of Hydrolysis

There are two types of enzymes used in modifying food proteins. One is endopeptidase cleaving peptide bonds from inside and the other is exopeptidase cleaving terminal groups at either amino or carboxyl end of peptide chains. Most enzyme-treated food proteins are the endopeptidase-treated products. The extent of enzyme treatment was measured by degree of hydrolysis (DH) based on the STNBS assay previously described.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

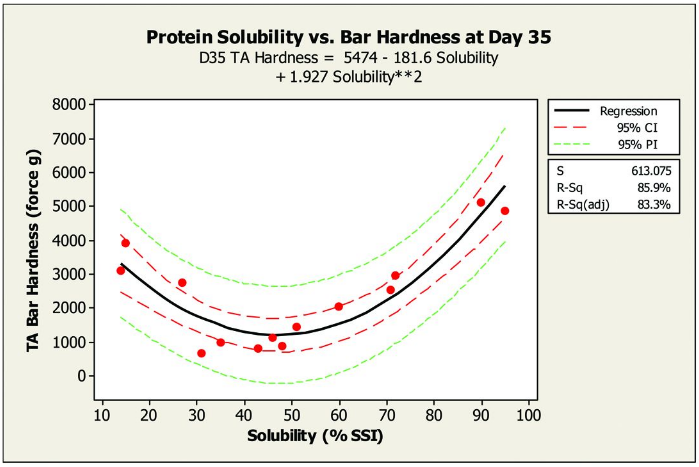


Figure 3. Solubility vs. Mechanical Bar Hardness at Day 35 in the 30% Protein, All Soy, All Sugar Syrup Formulation

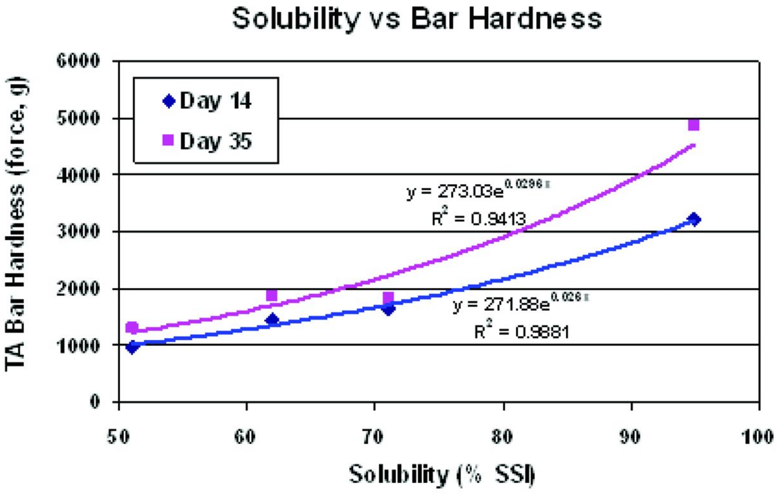


Figure 4. Mechanical Bar Hardness of Enzyme-Treated Proteins as a Function of Solubility and Storage (Day 14 and Day 35) in the 30% Protein, All Soy, All Sugar Syrup Formulation

This assay measures total number of terminal amino groups available in a protein, and the DH is calculated based on the increased number of terminal amino groups from that of intact protein with no enzyme treatment and the theoretical total number of amino acid residues at 100% DH (885 moles per 100 kg of soy protein). The intact soy protein has about 24 moles of terminal amino groups per 100 kg of protein primarily from the ε-amino groups of lysyl residues. The DH is significantly affected by the hydrolysis condition such as enzyme dose, temperature, time, mixing and substrate concentration. Protein undergoes functional and molecular changes during the enzyme treatment. Among these, solubility and viscosity are affected most. Solubility of soy protein at neutral pH decreases initially and then increases as the DH increases to high range. On the other hand, viscosity decreases as the DH increases, and then it eventually levels off. The molecular weight decreases with the DH increase. Solubility, viscosity and molecular profile within a protein are inter-related to each other, and they seem to be very critical parameters to interaction with water. The intact protein with both high solubility and high viscosity consumes the most water and made the worst bars. The enzyme-treated proteins produced food bars with various textures depending on their solubility and viscosity ranges associated with their DH. Therefore, it is difficult to demonstrate pure DH effect on the bar hardness even within the enzyme treated proteins.

Regardless, the DH showed a good correlation with the bar hardness, a similar trend to that of the solubility in the 30% protein all soy and sugar syrup bar formulation. The bar hardness increased as the DH increased at beyond 50 STNBS or decreased from the optimum at around 45 STNBS (Figure 5a, R^2 = 0.76). The bar chewiness increased as the DH increased (Figure 5b, R^2 = 0.82). The bar dough made with higher DH proteins tended to be soft but more sticky and their respective bars became more chewy over time than those of the lower DH proteins.

However, within the same product line with high DH range (≥ 75 STNBS), the bar hardness decreased as DH increased (Figure 6). Two sample series tested in the 30% protein all soy, all sugar syrup formulation showed significant correlations between DH and bar hardness. In these high DH and very low viscosity proteins, the solubility seemed to be no longer the dominating factor, but other parameters such as hygroscopic property acquired by the high DH might have impact on the bar texture.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

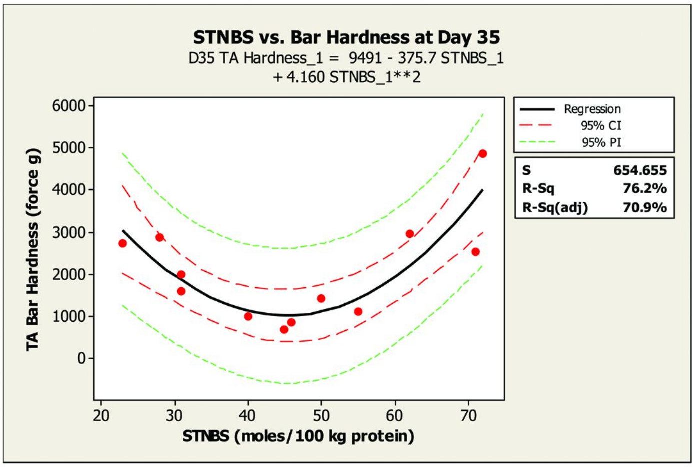


Figure 5a. DH vs. Mechanical Bar Hardness (force, gram) of the 30% Protein, All Soy, All Sugar Syrup Formulation at Day 35

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

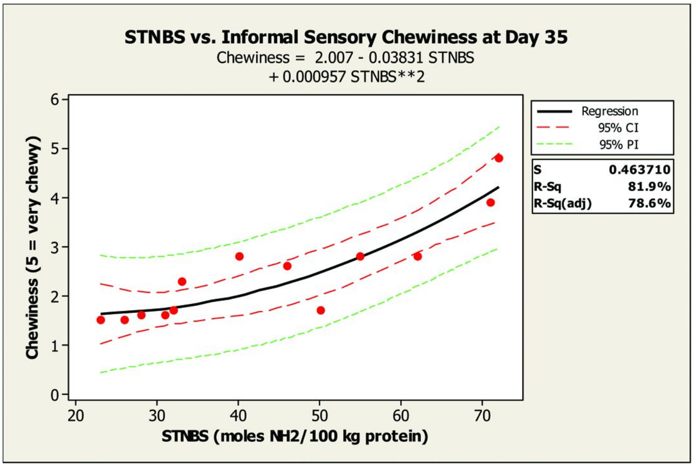


Figure 5b. DH vs. Chewiness Measured by Informal Sensory Panel for the 30% Protein, All Soy, All Sugar Syrup Formulation at Day 35.

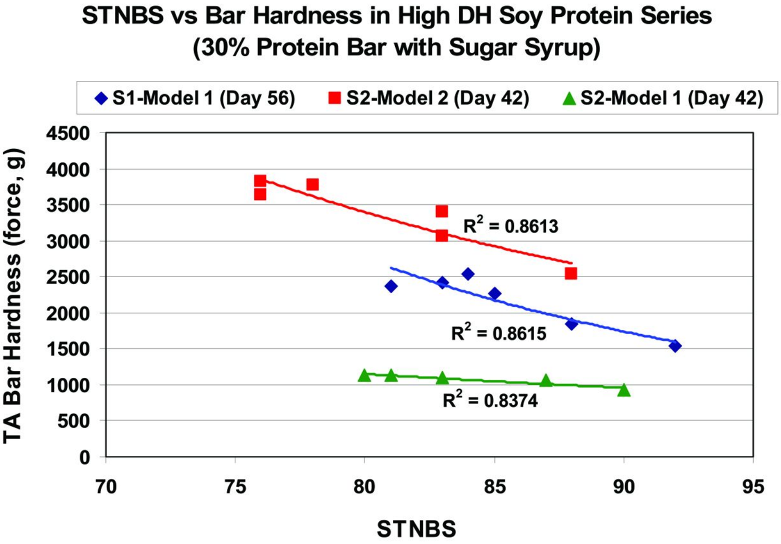


Figure 6. Mechanical Bar Hardness of High DH Soy Protein Series at Day 56 or Day 42. Two sample sets (S1 and S2) were evaluated in the 30% protein all soy sugar syrup formulation with different processing conditions (Model 1 and Model 2).

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Effect of Particle Size

The particle size effect on bar hardness was observed for the first time when two proteins with similar solubility and DH but different particle size were compared in the bar application test. This indicates that particle size is a secondary parameter surfacing after the two primary properties (solubility and DH) are controlled to the optimum ranges. The protein with larger particle size yielded softer bars than that with smaller particle size. The particle size effect was clearly demonstrated by a grinding experiment of Supro® Nuggets 311 product. The nuggets were ground to two different particle size ranges as "Coarse Ground" and "Fine Ground". The particle size profiles of these two are compared in Figure 7. The Coarse Ground showed 16% on 100 mesh screen (150 micron opening), 66% on 325 mesh (45 micron) and 73% on 400 mesh (37.5 micron). The Fine Ground had 0.3% on 100 mesh, 14% on 325 mesh and 29% on 400 mesh. As expected, the Coarse Ground sample produced much softer bar than the Fine Ground (Figure 8). Further, the particle size effect was observed in some commercial ISP products with similar solubility. For example, Supro® 320 with 53 µm mean particle size produced significantly softer bars (1250±38g at 35 days) than Supro® 661 with 33 µm mean particle size (2185±76g). The protein powder with larger particle size has smaller surface area than that with smaller particle size and would absorb less water from liquid ingredients. This leaves more water available in the bar matrix contributing to softer texture.

Effect of Density

Bulk density of ISP powder is related to other physical properties such as solubility and particle size. In general, the intact soy protein with higher solubility has lower density than a hydrolyzed protein with lower solubility. Also, ISP powder with larger particle size has lower density than that with smaller particle size. The typical range of bulk density for commercial spray-dried ISP powders is 0.20-0.35 g/cc. The density effect on the bar hardness was discovered when some ISP samples with similar solubility and particle size produced food bars with various hardness. Later, this was found to be related to their different densities as shown in Figure 9. Protein powders with higher density produce softer bars ($R^2 = 0.97$). The density appears to affect the bar hardness through a different mechanism from the others, i.e. physical versus the chemical interaction involving water in the others. The bar process requires mixing powder ingredients, mainly protein source, with limited amounts of liquid ingredients, mainly carbohydrate source such as sugar syrup. No free water is used in the typical bar formulation. Therefore, it is very critical to mix the powder ingredients with sugar syrup before it becomes crystallized. The high density powders are physically easier and more quickly mixed in than the light density powders, producing more moist and softer bars.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

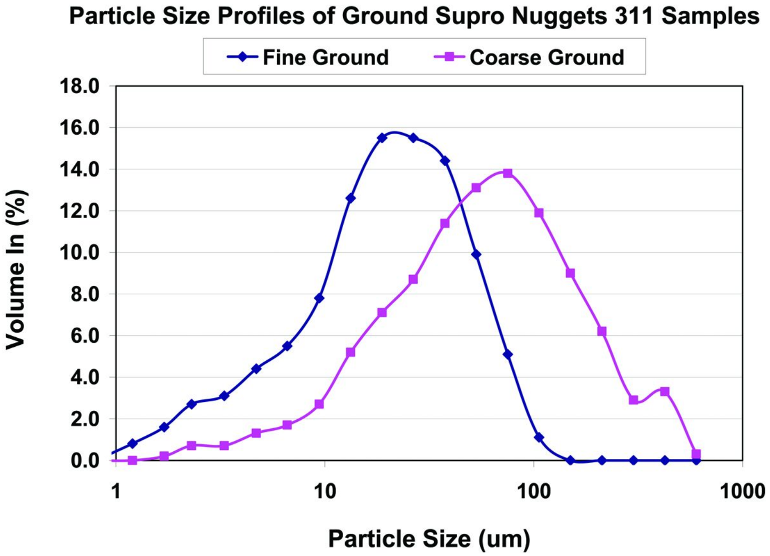


Figure 7. Particle Size Profiles of Ground Supro® Nuggets Measured by Malvern in Volume Distribution

Effect of Particle Morphology

Particle surface morphology was examined when two experimental ISP samples with very similar solubility, DH, particle size and density produced different texture bars. Based on the scanning electron microscopy (SEM) characterization (Figure 10), three morphology types were identified. 1) smooth large and small deflated spherical particles associated with intact soy protein, 2) cracked egg shell type fragments of smooth surface associated with enzyme-treated product, and 3) shriveled surfaces of many convolutions without collapse, i.e. “puffy” particle morphology. The ISP product with the third type surface morphology was found to produce softer texture bars in the 30% protein, all soy, all sugar syrup bar formulation.

Bar Functionality of Commercial Isolated Soy Proteins

Synergistic Effect of Two Soy Protein Blends on Bar Hardness

Supro® 320 with high molecular weight (MW) and intermediate solubility (45-55% SSI) produced acceptable bar hardness with short texture in the 30% protein with all soy sugar syrup formulation (*8*), but it did unacceptable hard texture in the high protein formulation (≥30% protein) of soy-dairy with sugar alcohols (polyols). On the other hand, Supro® 313 with low MW and high solubility (80-90% SSI) produced hard texture in the all soy sugar syrup

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

formulation, whereas it did soft texture in the high protein, reduced or no sugar syrup formulation with polyols.

However, combining Supro® 320 and Supro® 313 together at various ratios showed a synergistic effect on the bar hardness in the 30% protein, all soy sugar syrup formulation. The mechanical bar hardness data of the Supro® 320/313 blends at 100/0 to 0/100 ratios are shown in Figure 11.

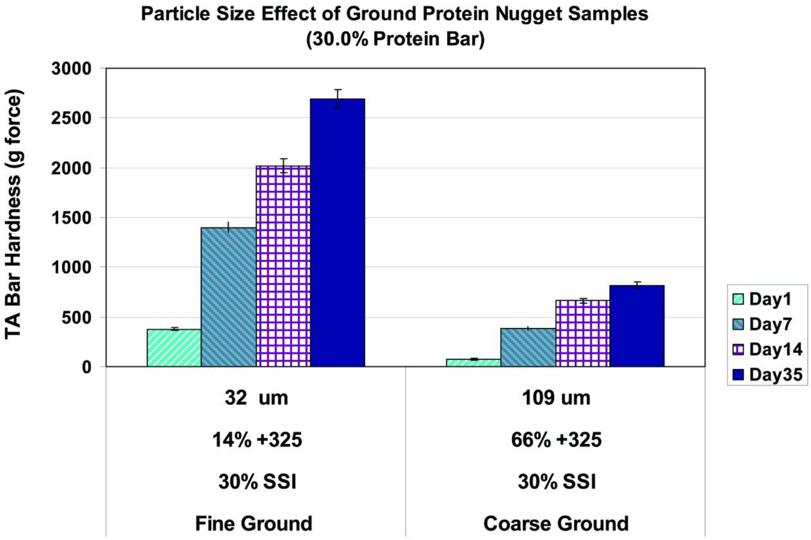


Figure 8. Mechanical Bar Hardness of Ground Supro® 311 Nuggets at Day 1 to Day 35: Fine Ground (left) and Coarse Ground (right). Mean particle sizes measured by two methods (Alpine sieve analysis with 325 mesh and Malvern).

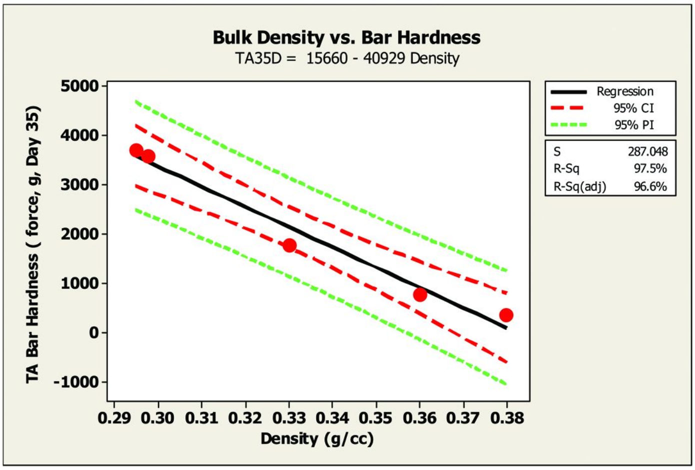


Figure 9. Mechanical Bar Hardness as a Function of ISP Powder Bulk Density

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

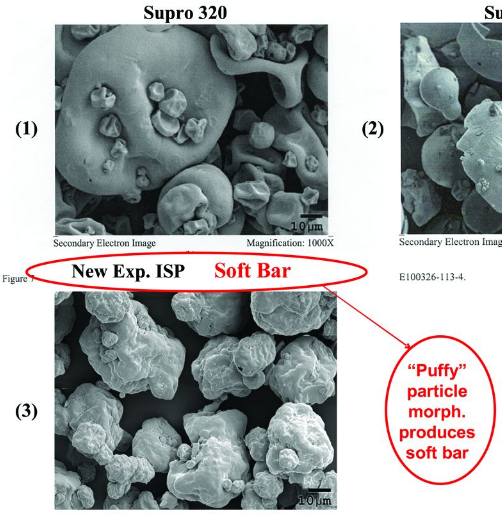

Supro 320
Supro 313
(1)
(2)
10μm
10μm
Secondary Electron Image
Magnification: 1000X
Secondary Electron Image
Magnification: 1000X
(b)
Figure 7
New Exp. ISP
Soft Bar
E100326-113-4.
(3)
10μm
"Puffy" particle morph. produces soft bar

Figure 10. SEM Images of Various ISP Products. (1) Supro® 320, (2) Supro® 313, (3) Experimental ISP

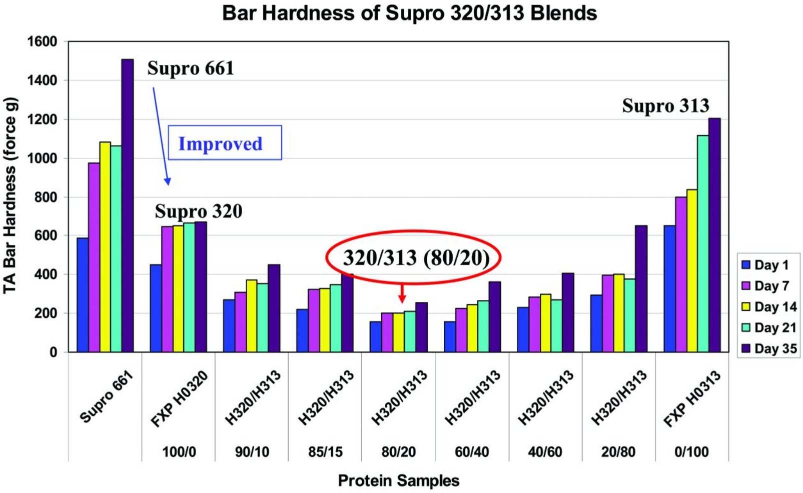


Figure 11. Mechanical Bar Hardness as a Function of Storage for Various Supro® 320/313 Blends in the 30% Protein All Soy, All Sugar Syrup Bar Formulation. Supro® 661 was included as a baseline reference. (see color insert)

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

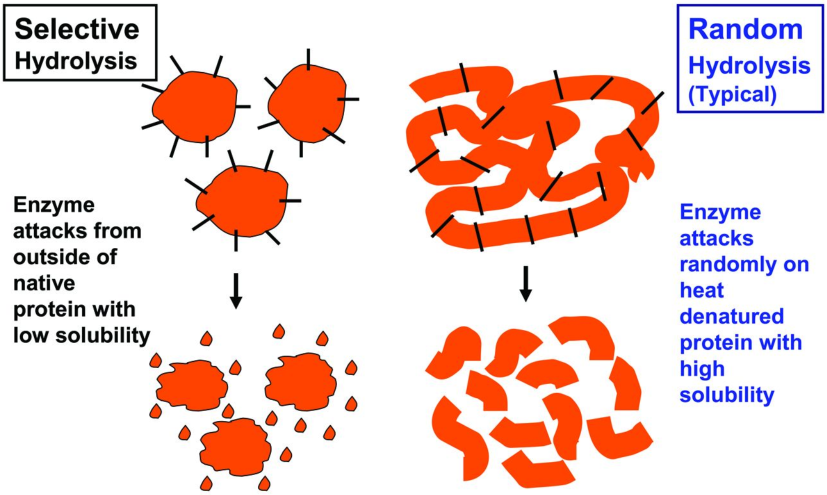

Selective Hydrolysis
Enzyme attacks from outside of native protein with low solubility
Random Hydrolysis (Typical)
Enzyme attacks randomly on heat denatured protein with high solubility

Figure 12. Type of Hydrolysis with Endopeptidase. "Selective" hydrolysis on left and "Random" hydrolysis on right

All blends produced softer bars (255-652g) than each protein alone (669g for Supro® 320 and 1205g for Supro® 313). Among the various Supro® 320/313 blend ratios, the 20% inclusion of Supro® 313, i.e. the 80/20 blend of the Supro® 320/313, produced the softest bar (255g) and the bar hardness increased in either sides. Here, Supro® 320 seems to play a "structure" protein providing short texture, whereas Supro® 313 plays a "binding" protein through its cohesive nature providing soft-chewy texture and binding function for other powder ingredients in the bar formulation (*8*). The mechanism of this synergistic effect of the Supro® 320/313 blend on the bar hardness is not well understood. Supro® 320 being an intact soy protein with high water holding capacity produced food bars with moderately hard and short texture, whereas Supro® 313 with a high DH and solubility produced food bars with sticky and soft texture initially but becoming hard during storage. These two different proteins probably have a unique complementary effect, interfering with each other's property. Supro® 313 also showed a synergistic effect with dairy proteins such as caseinates and whey proteins in a high protein soy-dairy bar formulation (*6*). Supro® 320 is very similar to Supro® 661 in most properties except for its larger particle size. This particle size effect is shown in the softer texture of the bars made from Supro® 320 compared to those from Supro® 661 (Figure 11).

Single Protein Solution for Diverse Bar Formulations

Protein Characterization

A technical hypothesis for a single protein solution was derived based on the Supro® 320/313 blend result: An ideal protein composed of mainly large MW fractions with smaller proportion of small MW fractions would produce soft bars in the designated bar formulation. Such an ISP product, Supro® 430, was successfully developed under a defined hydrolysis process using an endopeptidase and post-hydrolysis process conditions for optimum solubility, highest density and largest particle size possible (*9*).

The "Selective" hydrolysis designed for Supro® 430 is hypothetically compared to "Random" hydrolysis which is the typical hydrolysis option for food proteins in Figure 12. The "Selective" hydrolysis is using a relatively insoluble intact soy protein substrate, i.e. globular protein without denaturation (opening up molecules), therefore the enzyme cleaves protein molecules from outside peptide bones of the soy globular structure. In this case, the finished protein product would contain both high MW and low MW protein fractions providing both "structure" and "binding" functionalities in the bar formulation. On the other hand, the "Random" hydrolysis is based on the unfolded protein substrate denatured by heat treatment. In this case, the enzyme cleaves the protein molecules randomly throughout exposed peptide chains. These two different types of hydrolysis were demonstrated by SDS-PAGE and SEC-HPLC protein molecular profile data. The two sample sets of the Selective and Random hydrolysis with the same DH (STNBS) values showed distinctly different PAGE patterns (Figure 13). The Random hydrolysis samples (Random ISP1 & ISP2) were significantly more hydrolyzed than the Selective hydrolysis samples (Select ISP1 &ISP2). The former had no intact 7S and 11S proteins (major storage proteins in soy), whereas the latter still showed intact 11S protein. The SEC profiles also were differentiated between the two types of hydrolysis (Figure 14). The Selective hydrolysis samples have uniquely identifiable peak at around 25 minutes elution time or 43-55 Kd molecular weight region. This molecular peak has served as a marker for checking reproducibility of the Selective hydrolysis process. The exact nature of the cleaved proteins is not known. In addition, the Selective hydrolysates showed the desirable particle morphology that produced soft texture aforementioned (Figure 10). The protein particles had shriveled surfaces with many convolutions (Figure 15). Furthermore, these Selective hydrolysates achieved the optimum solubility (34-38% SSI) and high density (0.45-0.48 g/cc) ranges.

Reproducibility of the Selective hydrolysis process was demonstrated in both pilot and commercial scales. Supro® 430 produced at a commercial scale had the optimum solubility, high density and desirable particle morphology, and made food bars with desirable texture as discussed later.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Performance in Bar Application

The mechanical bar hardness values of various experimental protein samples tested in the 30% protein all soy, all sugar syrup formulation measured by Texture Analyzer (TA) are compared in Figure 16. The Selective hydrolysis proteins (Select ISP1 and ISP2) with both large and small molecular weight fractions yielded bars maintaining softer texture over shelf life than those of the Random hydrolysis (Random ISP1 and ISP2) with mainly medium molecular weight fractions. This proved the importance of the type of hydrolysis in the bar proteins. Also, the bar hardness data of Select ISP1 & ISP2 comparing to the Supro®

320/313 blend (Figure 16) clearly demonstrated that the designed protein via the Selective hydrolysis delivered the bar functionality expected. These Selective hydrolysates appear to provide a good combination of large MW fraction for bar structure (like Supro® 320) and small MW fraction for binding function (like Supro® 313) in the bar formulation.

Supro® 430 batches produced at a commercial scale were tested in two bar formulations to demonstrate unique and diverse bar functionality of the Selective hydrolysis product. One was the 30% protein all soy all sugar syrup formulation comparing with the Supro® 320/313 blend, and the other was the high protein no sugar formulation with 40% protein of soy-dairy (33% soy, 33% calcium caseinate, 33% WPC) comparing with Supro® 313.

As discussed, Supro® 320 functioned well in the all soy low protein bar formulations, but not in the 30% or higher protein bars with or without reduced sugar syrup. On the other hand, Supro® 313 functioned well in the high protein of soy-dairy with no or reduced sugar syrup models, but not in the high protein all sugar syrup bar formulations. The Supro® 320/313 blend functioned well and provided diverse texture in the 30% protein all soy all sugar syrup formulation. In contrast, Supro® 430 functioned well in both the 30% protein all soy all sugar syrup, and the high protein with no or reduced sugar syrup formulations (Figures 17 and 18). The bar hardness at 42 days at 32°C was determined to be equivalent to 12 months at ambient temperature.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

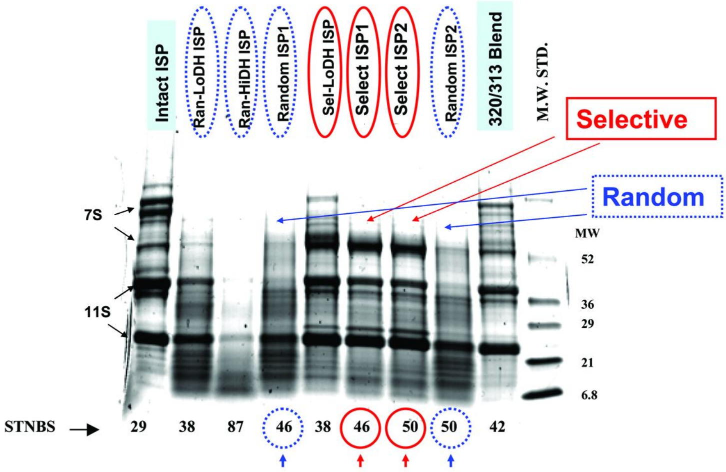


Figure 13. SDS-PAGE of Two Types of Hydrolysis. "Selective hydrolysis" samples in solid circle and "Random hydrolysis" samples in dot circle. Note the different band profiles of two sets of the selective and random hydrolysis samples with same STNBS values, 46 (Select or Random ISP1) or 50 (Select or Random ISP2). Other samples are a low and high DH ISP from the random (Ran-LoDH, Ran-HiDH) or selective hydrolysis (Sel-LoDH ISP) (see color insert)

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

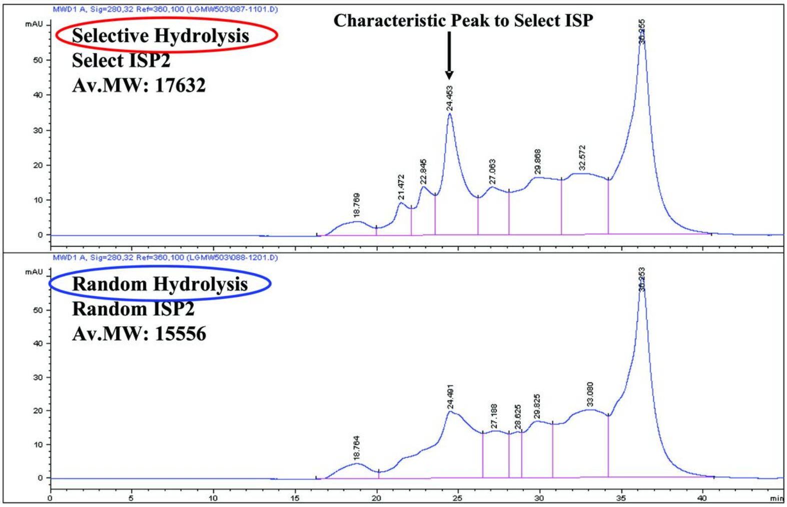


Figure 14. Size Exclusion Chromatography HPLC Molecular Weight Distribution Profiles: Select ISP2 from the selective hydrolysis vs. Random ISP2 from the random hydrolysis with the same degree of hydrolysis

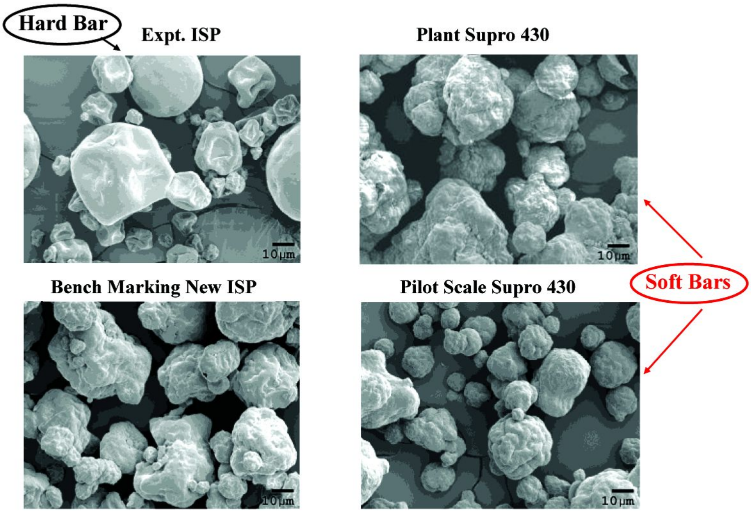

Hard Bar
Expt. ISP
Plant Supro 430
Bench Marking New ISP
Pilot Scale Supro 430
Soft Bars
10μm
10μm
10μm
10μm

Figure 15. SEM Images of Various Experimental ISP Samples. The Supro® 430 samples showed similar morphology to the bench-marking experimental protein with the desirable morphology identified

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

Supro® 430 outperformed the 80/20 blend of Supro® 320 and 313 in the 30% protein all sugar syrup formulation, producing a significantly softer texture than the blend (Figure 17). Supro® 430 produced comparably soft bars to Supro® 313 in the high protein no sugar syrup formulation (Figure 18). The Supro® 320/313 blend yielded a very hard bar in this system (data is not shown). In addition, Supro® 430 reduced bar dough stickiness significantly compared to Supro® 313 (Figure 19). Reduced dough stickiness improves bar manufacturing efficiency from easier processing and cleaning.

Li, et al (*6*) used NMR state diagram to categorize various proteins in powder form including Supro® 320, Supro® 313, Supro® 430 and dairy proteins into four groups. The NMR state diagrams were analyzed in relation to bar textural properties of three high protein bar formulations, i.e. all sugar, reduced sugar, and no sugar syrup with polyols, during accelerated storage. Based on this, Supro® 313 and Supro® 430 belong to Group 1 which is predicted to be most stable undergoing few changes during storage. Supro® 320 is in the next stable Group 2. On the other hand, the dairy proteins tested (milk protein isolate, whey protein isolate and calcium caseinate) belong to less stable Group 3 or 4. Supro® 313 and Supro® 430 provided the most diverse and synergistic effect when they were blended with other proteins, particularly dairy proteins.

The general trends of soy protein properties related to food bar texture based on two bar formulations are summarized in Table 2. This should be used only as a guideline.

Table 2. Summary of Soy Protein Properties vs. Bar Functionality

Soy Protein Properties	***30% Protein All Soy, All Sugar Syrup***	***35-40% Protein/Low Carb. 33-50% Soy/Dairy Proteins***
Degree of Hydrolysis (DH)	-Higher DH, harder bar, more chewy, more sticky	-Higher DH, softer bar, more chewy, more sticky, more bitter
Solubility	-Higher solubility, harder bar, more chewy -Too low solubility, hard and dry bar -Optimum at 30-40% NSI*	-Higher solubility, softer bar, more chewy -Higher solubility, more sticky
Density	-Higher density, softer bar, easier to process	-Higher density, softer bar, easier to process
Particle Size (PS)	-Larger PS, softer bar	-Larger PS, softer bar

* NSI: Nitrogen Solubility Index

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

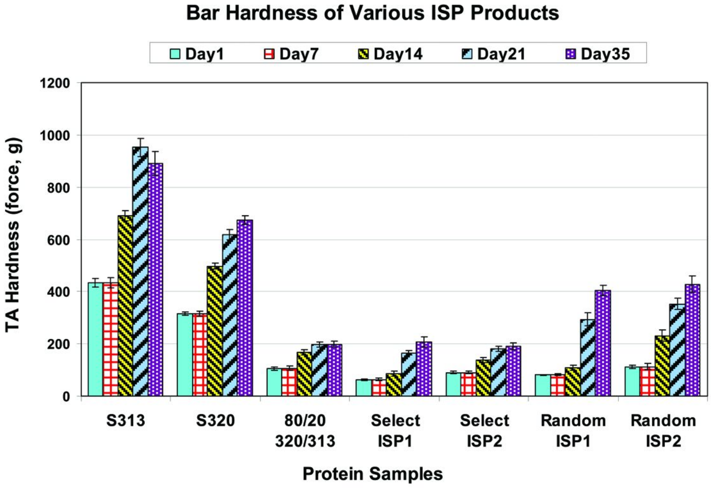


Figure 16. Mechanical Bar Hardness as a Function of Storage for Various Soy Proteins in the 30% Protein All Soy, All Sugar Syrup Bar Formulation: Supro® 313, Supro® 320, the 80/20 blend of Supro® 313 and Supro® 320, two proteins from the selective hydrolysis (Select ISP1 & ISP2) and two proteins from the random hydrolysis (Random ISP2 & ISP2)

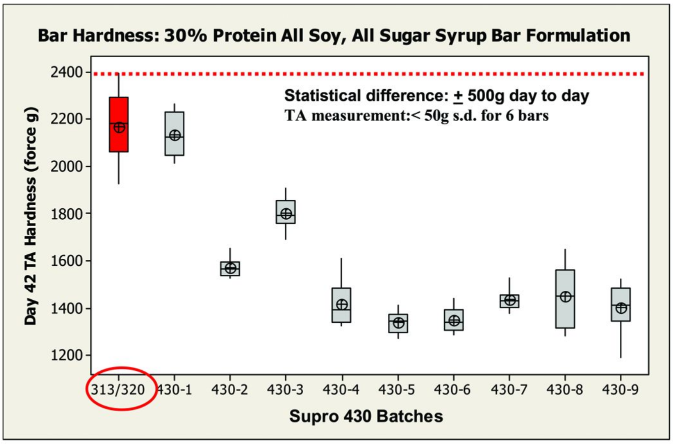


Figure 17. Mechanical Bar Hardness of Supro® 430 Batches Compared to the Supro® 313/320 Blend in the 30% Protein All Soy, All Sugar Syrup Formulation at Day 42 under the Accelerated Storage at 32°C.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

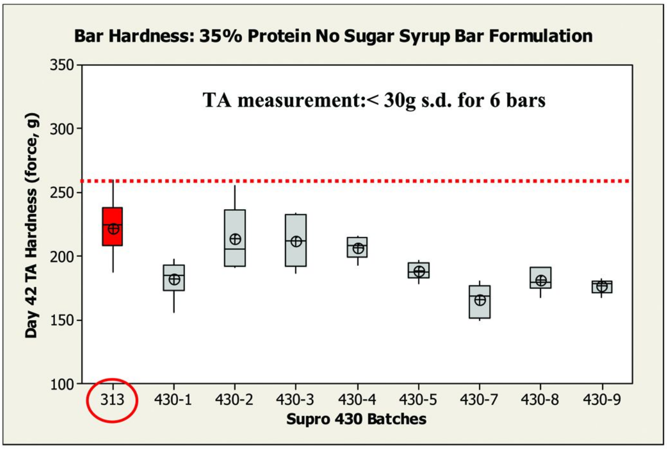


Figure 18. Mechanical Bar Hardness of Supro® 430 Batches Compared to Supro® 313 in the 35% Protein, 33% Soy, No Sugar Syrup Formulation at Day 42 under the Accelerated Storage at 32°C

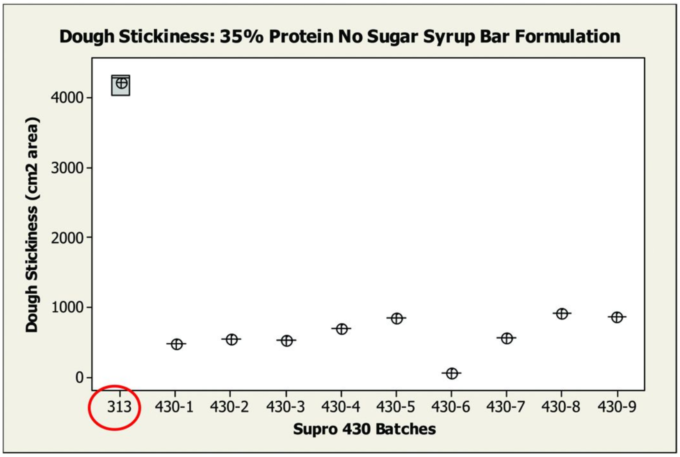


Figure 19. Bar Dough Stickiness of Supro® 430 Compared to Supro® 313 in the 35% Protein, 33% Soy, No Sugar Formulation

Conclusions

The mechanical hardness of food bar measured with Texture Analyzer was found to be highly correlated with the hedonic Overall Liking score in sensory evaluation of the high protein food bars. Five critical properties of soy protein that influenced bar hardness over time were identified: protein solubility, degree and type of enzymatic protein hydrolysis, density, particle size and particle surface morphology. Protein solubility and degree of hydrolysis are the primary protein properties most significantly affecting the bar hardness and sensory. Density is the secondary property appearing after the primary properties are controlled to desirable range. Particle size effect is shown only when the primary and secondary properties are optimized. Commercially available isolated soy proteins, Supro® 320, Supro® 313 and Supro® 430 can provide technical solutions for creating desirable texture and shelf life in the high protein food bar formulations containing various dairy proteins and sugar syrup levels.

Acknowledgments

This research was greatly supported by Steven Taillie, Ken Bopp, Tom Wagner and Linda Northrop who provided the bar application work for many experimental and commercial ISP samples. Special thanks are directed to Charlie Kolar, Andreas Altemueller and Ted Wong who provided invaluable comments on the manuscript.

Downloaded by STANFORD UNIV GREEN LIBR on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch019

References

1. *Recommended Dietary Allowances*, 10th ed.; National Research Council: Washington, DC, 1989; pp 56−57.
2. Food Labeling: Health Claims; Soy Protein and Coronary Heart Disease, FDA, 21 CFR Part 101, *Fed. Regist.*, **1999**, *64*, (206), FR 57699.
3. Taillie, S. A. Food Bars. In *Soy Applications in Food*; Riaz, M. N., Ed.; CRC Press, Taylor & Francis: Boca Raton, FL, 2005; pp 185−197.
4. Michnowski, J. A. Process for Making a Nutritional Bar. U.S. Patent 4,543,262, 1985.
5. Painter, J. E.; Prisecaru, V. I. The effect of various protein and carbohydrate ingredients in energy bars on blood glucose levels in humans. *Cereal Foods World* **2002**, *47*, 236–241.
6. Li, Y.; Szlachetke, K.; Chen, P.; Lin, X.; Ruan, R. Ingredient characterization and hardening of high-protein food bars: An NMR state diagram approach. *Cereal Chem.* **2008**, *85* (6), 780–786.
7. *GE Six Sigma Quality Coach*, version 1; General Electric Company, 2001.
8. Taillie, S. A.; Cho, M. J. Soy Protein Blend for Soft Confectionery Food Bars. U.S. Patent 7,419,695 B1, 2008.
9. Cho, M. J. Isolated Soy Protein Having High molecular Weight Protein Fractions and Low Molecular Weight Protein Fractions. U.S. Patent Application 2007/0042103 A1, 2007.
10. Fields, R. The Rapid Determination of Amino Groups. In *Methods of Enzymology*; Kaplan, N. O., Ed.; Academic Press: New York, 1972; Vol. 25, Part B.
11. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophase T4. *Nature* **1970**, *227*, 680–685.
12. Hames, B. D.; Rickwood, D., Eds.; *Gel Electrophoresis of Proteins: A Practical Approach*; Oxford University Press: New York, 1981.
13. Lin, X.; Ruan, R.; Chen, P.; Chung, M.; Ye, X.; Yang, T.; Doona, C.; Wagner, T. NMR state diagram concept. *J. Food Science* **2006**, *71* (9), 136–144.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Chapter 20

Sensory Evaluation Techniques To Promote Extruded Soy Foods Consumption and Increase Consumer Acceptance

Soo-Yeun Lee,*[,a] Erika A. Neely,[a] and Youngsoo Lee[a]

[a]Department of Food Science and Human Nutrition,
University of Illinois at Urbana-Champaign, Urbana, IL 61801
***soolee@uiuc.edu**

Healthy extruded soy foods are of interest to many researchers and food companies, and have therefore been evaluated extensively using a variety of sensory techniques. The literature shows that although soy snacks and cereals have generally had trouble gaining marketshare in the past due to poor consumer acceptance, some recent attempts have been able to better understand the drivers of liking for extruded soy foods, thereby allowing a directed approach to improving the products. By applying the appropriate sensory evaluation techniques, such as preference mapping and conjoint analysis, one can tailor the products to a specific target consumer group and achieve strong consumer acceptance.

Soy Foods

Health Benefits of Soy

Soy foods have been shown to possess a multitude of health benefits, including providing high-quality protein, exhibiting hypocholesterolemic and anticarcinogenic effects, and promoting bone and kidney health (*1*). In developing countries where protein-energy malnourishment is an ongoing problem, particularly in children, supplemental foods that provide an inexpensive source of high quality protein would benefit at-risk populations (*2*).

Many attempts have, therefore, been made to create food products designed to deliver soy to populations requiring nutritional intervention. Particularly,

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

many researchers have demonstrated the beneficial effect of consuming soy in combination with other grains due to their complementary amino acid profiles. Early research on supplemental foods for use in global food aid programs found that corn and soybean blends provide a better balance of essential amino acids (*3*). Subsequently, organizations such as UNICEF and the Food For Peace programs began distributing corn-soy-milk (CSM), corn-soy blend (CSB) and wheat-soy blend (WSB) products to developing countries (*4*, *5*). Since that time, efforts have produced a variety of food products containing corn and soy with protein quality shown to be similar to that of milk (*6*, *7*). Likewise, rice and soy have been combined to produce foods that deliver protein quality superior to that of rice or soy alone (*8–11*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Soy Extrusion and Soy Snacks

One of the more well-studied methods for manufacturing soy-containing food products is extrusion cooking. Extrusion is an efficient method for producing uniform food products, and is, therefore, an attractive option for large scale supplementation operations for developing countries (*12*). Furthermore, the high temperature and pressure processing that extrusion can provide has been shown to reduce anti-nutritional factors, like trypsin inhibitors found in raw soybeans (*4*, *13*).

As extrusion is commonly used to produce ready-to-eat (RTE) breakfast cereal and snack foods, it comes as no surprise that many have studied soy as an ingredient in snacks and cereal products. Generally, soy ingredients appear to have the largest effect on texture of the products, with flavor also being affected. However, the choice of ingredients and parameters used to make the products vary widely across studies, leading to differing conclusions amongst researchers as to the effects of soy ingredients on the extruded products. Addition of protein has been cited to reduce puffing in extruded cereal and snack applications (*14*). This principle was observed experimentally by Park (*15*) in a rice snack product containing 20-30% soy flour. The researcher concluded that the inability of protein to swell like starch when heated reduced the friction energy and thus the amount of starch gelatinization. Adesina and others (*16*) also reported that snacks made from defatted soy flour and corn flour in various ratios exhibited a decrease in hardness and expansion as soy level increased, which is explained by formation of a fibrous protein structure during the extrusion process. However, Breen and others (*17*) and Camire and King (*18*) found the addition of SPI to high fiber corn-based snacks improved the product texture by increasing expansion. Still others reported that soy protein inclusion did not have an effect on extrudate properties. A fried corn-based snack containing up to 20% defatted soybean meal was found to be not significantly different in sensory attributes from an all-corn control (*7*). Similarly, Martinez-Flores and others (*19*) found that a puffed snack containing up to 17% soybean paste did not differ significantly in flavor or crunchiness compared to one made from 100% corn. Clearly, the effects are highly dependent on the formulations employed in a particular situation.

Sensory Evaluation of Soy Snacks and Cereals

Consumer Acceptance of Extruded Soy Foods

Products that are presented in familiar contexts or that have been determined to have acceptable sensory properties will have a better chance of being successfully incorporated into the diet (*20*). Therefore, evaluating consumer acceptance of novel soy snacks prior to their introduction to the target market is important for market success. As personal preferences vary widely across individuals, consumer acceptance tests typically employ a large group (ideally at least 75 consumers) selected from the target user group of the product in question, in order to help achieve a representative sample and to allow groupings of the consumers into meaningful segments (*21*). Although the consumers should be somewhat familiar with the product or category, they must be naïve to the nature of the test samples or the specific test objectives at hand in order to reduce response bias. Therefore, it is generally not recommended that the investigators or closely associated parties participate in acceptance testing (*22*). However, many of the studies examining soy snacks have not evaluated consumer acceptance using adequate testing methods. Nonetheless, their findings may be interpreted as guidance for further investigation.

In terms of the effect of soy amount on consumer acceptance, some found no significant differences among the samples in a variety of contexts: a fried snack with 20-40% soy flour (*11*, *19*, *23–25*), flake cereal containing 10-30% full fat soy flour (FFSF) (*11*), extruded snacks with 8-17% soybean paste (*19*), and pelleted supplemental foods containing 10-20% defatted soy flour (DFSF) (*24*). In contrast, others found that only relatively low levels of soy ingredient addition achieved products that were not significantly different from a reference product containing no soy, 10% partially defatted soy flour (PDSF) in one case (*26*), and 20% DFSF in another (*7*). Adesina and others (*16*) reported that a snack containing 25% soy was most acceptable, while Faller and others (*27*) found snacks containing 15% soy to be liked by the majority of consumers tested. In a rice-soy snack, up to 13.5% full fat soy flour (FFSF) was found to be acceptable (*9*). Yeu and others (*28*) showed that up to 54% soy in a breakfast cereal received neutral acceptance ratings and was not significantly different from the samples containing as low as 41% soy, concluding that relatively high soy levels may be acceptable to consumers. Neely (*29*) found that extruded soy snacks containing at least 52% soy were generally found to be acceptable by consumers familiar with Indian-style snacks. Of the studies mentioned here, only Neely (*29*), Yeu and others, (*28*), Baskaran and others (*24*) and Faller and others (*27*) conducted studies with over 100 panelists, so the other consumer samples may not be large enough to constitute representative samples of the target population. However, again, it appears that acceptability is largely dependent on the context, so it is difficult to generalize across studies. One notable trend across all of these studies, however, is that with the exception of Neely (*29*) and Yeu and others (*28*), none of the studies investigate products in which the majority (>50%) of the sample is composed of soy ingredients. Studies evaluating consumer acceptance of roasted soy nuts (*30*) and freeze-dried immature soybeans (*31*), both of which are 100%

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

soy products aside from small amounts of seasonings, generally found neutral to slightly positive acceptance scores for the tested samples.

Descriptive Analysis of Soy Snacks and Cereals

Descriptive analysis is a method that allows collection of objective sensory descriptions about products (*21*) . In other words, it provides profiles of products expressed in terms of their sensory characteristics, and therefore, allows comparison of products in terms of their sensory properties, much in the same way instrumental measurements might be used. Descriptive analysis requires a group of panelists to evaluate the products in an objective way, which requires training to ensure reproducibility and consistency (*21*).

In contrast to consumer testing, there are relatively few studies that have investigated soy-containing snacks and cereals using sensory descriptive analysis techniques. Nonetheless, their descriptive terminology and testing protocols may guide further descriptive profiling of extruded soy products. A breakfast cereal containing SPI was described by 19 sensory attributes spanning appearance, texture and flavor modalities (*32*, *33*). The terms can be seen in Table I. Differences across the samples were found at various sugar levels. The effects were also observed with varying feed moisture levels. A study on SPI and fiber-containing extruded snacks measured only 4 attributes (color intensity, hardness, air cell size and corn or grain flavor intensity) and found that samples containing SPI were more tan colored, less hard, contained larger air cells, and had more pronounced grain flavor. In an extruded snack containing minced catfish, corn and DFSF, the product was characterized by hardness, denseness, and strong grain complex flavor along with other less intense flavors of rice and dried grass (*34*). When garlic and onion were added to these snacks, associated garlic and onion flavors were perceived, although addition of ginger was not perceived. A series of Indian-style extruded soy snacks was found to vary across 14 sensory attributes (Table II), with soy level mostly affecting important flavor and texture attributes, and the use of soy grits contributing to color and taste changes (*29*).

Correlating Descriptive and Consumer Data for Soy Snacks and Cereals

Although consumer acceptance and descriptive analysis data each provide valuable information about products, correlating the two types of data allows even more powerful analysis by gaining an understanding for what specific sensory attributes contribute to hedonic responses. External preference mapping is one technique that can integrate these two types of data (*35*). This method allows descriptive analysis conducted by a trained panel of judges to form the framework upon which the consumer hedonic scores are projected, thus identifying sensory attributes that most influence liking (often termed “drivers of liking”) without relying on untrained consumer judgments of attributes. This technique has seen recent use with a variety of products including chocolate milks (*36*), cheddar cheeses (*37*), and sausages (*38*).

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Table I. Descriptive terms, definitions and references for descriptive sensory evaluation of corn/soy breakfast cereal. Used with permission

Dry Sample Characteristics	
Expansion	Degree of expansion in terms of the volume of a piece (7.5*=Kix; 14.5=Cocoa Blasts)
Surface Smoothness	Degree to which the surface is even or smooth (2.0=Cocoa Blasts; 14.5=Kix)
Hardness	Degree of force required for cutting samples (2.0=Kix; 14.5=Grape-Nuts)
Crunchiness	Degree of snap, as measured by noise, force and denseness, released from the sample (4.0=Cheerios; 14.5=Grape-Nuts)
Adhesiveness	Degree to which the mass sticks to teeth (7.5=Cocoa Blasts; 12.0=Kix)
Toasted Corn Flavor	Intensity of toasted corn flavor (14.5=Corn Flakes in milk)
Toasted Soy Flavor	Intensity of toasted soy flavor (14.5=Dried texturized vegetable protein)
Sweetness	Intensity of sweet taste (14.5=Frosted Cheerios)
Bowl Life (Wet Sample) Characteristics	
Initial Hardness	Degree of force required for cutting samples (2.0=Kix; 14.5=Grape-Nuts)
Denseness	Compactness of cross section of sample after biting completely through with the molars (1.0=Kix; 14.5=Grape-Nuts)
Toasted Corn Flavor	Intensity of toasted corn flavor (14.5=Corn Flakes in milk)
Toasted Soy Flavor	Intensity of toasted soy flavor (14.5=Dried texturized vegetable protein)
Sweetness	Intensity of sweet taste (14.5=Frosted Cheerios)
Wet Hardness	Degree of force required for cutting samples (2.0=Kix; 14.5=Grape-Nuts)
Crunchiness	Degree of snap, as measured by noise, force and denseness, released from the sample (4.0=Cheerios; 14.5=Grape-Nuts)
Moisture Absorption	Amount of moisture absorbed by the sample (1.0=Grape-Nuts, 14.5=Cheerios in milk for 4 minutes)
Adhesiveness	Degree to which the mass sticks to teeth (7.5=Cocoa Blasts; 12.0=Kix)
Residual Soy Flavor	Intensity of toasted soy flavor left after swallowing (14.5=Dried texturized vegetable protein)
Off-flavor	Intensity of unpleasant and unexpected residual flavor in the mouth

*intensity scores for references are 0=none to 15.0=extreme

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Table II. Descriptive terms, and associated references as generated by a trained descriptive panel evaluating soy snack foods. Where appropriate, all references were prepared with spring water as a solvent. Used with permission

Modality	*Term*	*Reference Preparation*	*Reference Product*
Appearance	Rough	1 cracker served in 59 mL cup	Schnucks Wheat Crackers (Schnuck Markets, Inc.; St. Louis, Mo.)
	Uniform Shape	5 pieces elbow macaroni, boiled for 6 minutes, served in 59 mL cup	Barilla® Elbows (Barilla America, Inc.; Bannockburn, Ill.)
	Dark	Paint sample card	Behr Light Copper paint (BEHR Process Corporation; Santa Ana, Calif.)
	Porous	1 chip served in 163 mL cup	Original Sun Chips (Frito Lay, Inc.; Plano, Tex.)
Aroma	Cumin	30 mL 0.4% w/w ground cumin infusion served in 59 mL cup	Ground Cumin (McCormick & Co., Inc.; Hunt Valley, Md.)
	Coriander	30 mL 0.4% w/w ground coriander infusion served in 59 mL cup	Ground Coriander (D'Allasandro Gourmet Ingredients, Gurnee, Ill.)
	Wheat	1 cracker served in 59 mL cup	Hint of Salt Wheat Thins (Kraft Foods Global, Inc.; Northfield, Ill.)
Texture	Brittle	2 "C" shaped pieces served in 59 mL cup	Fritos (Frito Lay, Inc.; Plano, Tex.)
	Crunchy	1 cracker served in 59 mL cup	Schnucks Wheat Crackers (Schnuck Markets, Inc.; St. Louis, Mo.)
	Crunchy	1 chip served in 59 mL cup	Tostitos® Bite Size Tortilla Chips (Frito Lay, Inc.; Plano, Tex.)
	Crunchy	2 pieces served in 59 mL cup	Cracklin' Oat Bran® (Kellogg Sales Co; Battle Creek, Mich.)

Continued on next page.

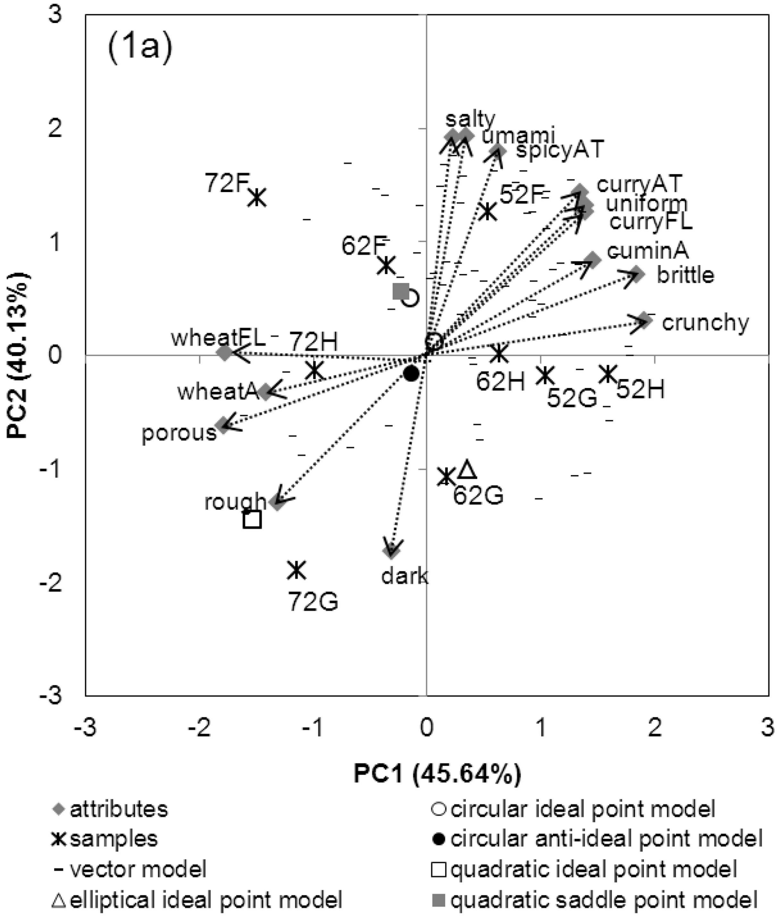
Table II. (Continued). Descriptive terms, and associated references as generated by a trained descriptive panel evaluating soy snack foods. Where appropriate, all references were prepared with spring water as a solvent. Used with permission

Modality	*Term*	*Reference Preparation*	*Reference Product*
Flavor	Wheat	1 cracker served in 59 mL cup	Schnucks Wheat Crackers (Schnuck Markets, Inc.; St. Louis, Mo.)
	Curry	20 mL 0.2% w/w curry powder infusion served in 30 mL cup	Curry Powder (McCormick & Co., Inc.; Hunt Valley, Md.)
	Cumin	20 mL 0.4% w/w ground cumin infusion served in 59 mL cup	Ground Cumin (McCormick & Co., Inc.; Hunt Valley, Md.)
Taste	Salty	20 mL 0.3% sodium chloride solution in spring water, served in 30 mL cup	Morton Salt (Morton International, Inc.; Chicago, Ill.)
	Umami	20 mL 0.4% monosodium glutamate solution in spring water, served in 30 mL cup	Aji-No-Moto Monosodium Glutamate (Ajinomoto U.S.A., Inc.; Portland, Oreg.)
Aftertaste	Curry	20 mL 0.15% w/w curry powder infusion served in 30 mL cup	Curry Powder (McCormick & Co., Inc.; Hunt Valley, Md.)
	Spicy	20 mL 0.25 ppm capsaicin solution in spring water with polysorbate 80 emulsifier, served in 30 mL cup	Capsaicin, USP (Spectrum Chemical Mfg. Corp.; Gardena, Calif.), Lumisorb PSMO-20 K (Lambent Technologies; Gurnee, Ill.)

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Recently, this technique has been applied to an extruded soy-based snack food to correlate sensory descriptive attributes with consumer acceptance in the U.S. and India (*29*). The results illustrated that for U.S. consumers, drivers of liking included salty and umami tastes, curry and cumin flavors, spicy aftertaste, crunchy texture and uniform appearance, while rough, dark and porous appearance, wheat aroma and flavor drove disliking (Figure 1a). Meanwhile, Indian consumers also preferred products with salty, umami and spicy attributes, but they tended toward products with stronger wheat flavor and porous appearance (Figure 1b). Thus, this study was able to illustrate distinct differences in preferences of the two populations that related to specific sensory attributes of the products at hand, thereby allowing future development efforts to drive consumer acceptance in an attribute-driven manner.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020



Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

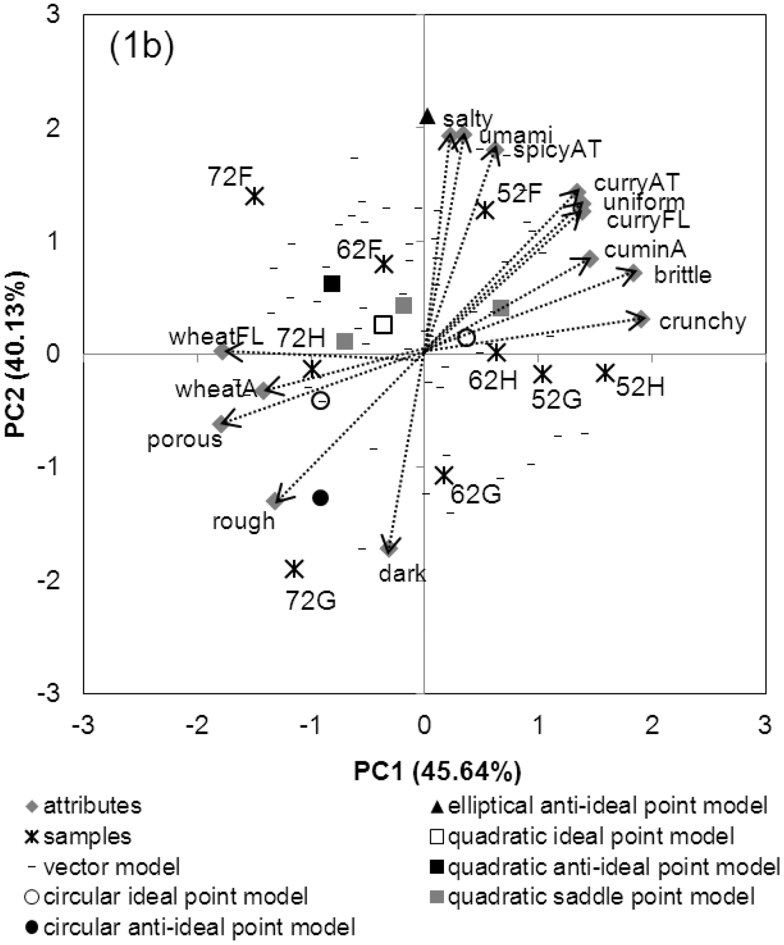


Figure 1. External preference maps of 9 extruded snack samples profiled by a trained panel and evaluated for overall liking by 72 U.S. (a) or 67 Indian (b) consumers. For attributes, A=aroma, FL=flavor and AT=aftertaste. For sample descriptors, 52, 62 and 72 indicate soy level (% dry formula weight) in a sample, and F, H and G indicate sample manufactured using soy flour, soy flour and grits blend, or soy grits, respectively. For each consumer, the marker indicates the model type that was found to best fit that consumer's preferences. Used with permission.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Soy Foods Product Development for Burgeoning International Markets

Although much of the research on soy foods has been conducted in the Western hemisphere, the purported benefits of soy foods consumption are not limited to Western populations. Therefore, there is an interest in increasing soy foods consumption globally, with a specific emphasis on regions that may not be receiving the maximum benefit offered by soy due to low consumption levels. However, in order to effectively promote new food products to culturally distinct populations, an examination of local traditions and dietary habits is necessary.

India Case Study

India, a country with over one billion inhabitants and economic growth attracting constant global attention, has become a prime focus for food companies trying to expand their markets to new regions. Therefore, a better understanding of Indian consumer attitudes, particularly in the context of differences from Western consumers, would contribute to successful targeting of products to this newly expanding market. However, there has been very little research comparing preferences of Indian and Western consumers. Moskowitz and others (*39*) highlighted perception and preference differences for intensely sour and bitter aqueous solutions by a group of Indian laborers in Karnataka, India, as compared to Indian medical students in Bangalore, India, and Western subjects from another study. The researcher concluded that, although the perception of the basic tastes did not differ much across the populations, dietary habits caused the laborer group to accept and prefer very sour or bitter foods. This conclusion is in agreement with the previously cited works on cross-cultural comparisons that found preference was not necessarily related to perceptual differences, but rather to habituation and acculturation. Nevertheless, more comparative research is necessary to better understand Indian consumer needs in the context of the global food economy.

Food preferences are primarily determined by one's exposure and familiarity to a food, which is, in turn, governed by cultural traditions (*40*). Therefore, in order to develop products that will appeal to Indian consumers, a basic understanding of traditional foods and habits is necessary. Although India is a vast and diverse country, some general commonalities across the nation will be cited here to give a brief overview of Indian cultural traditions.

In general, Indian food traditions are heavily governed by religious and spiritual beliefs, particularly Aryan (and the closely related Hindu) doctrines, and to a lesser extent other religions such as Buddhism, Sikhism, Jainism, Islam and Christianity, depending on the region (*41*). Although each religion touts its own set of dietary restrictions, many prescribe vegetarianism or limits on types of meat that may be consumed. Therefore, Indian meals often consist of a cereal such as rice or wheat complemented by legumes such as beans, lentils, green peas and chickpeas (*42*), in order to achieve adequate protein intake. These staples can be presented in a variety of forms, such as leavened or flat breads, pancakes, porridges, and stews (*43*). Indian cuisine is also characterized by the heavy use of spices to impart distinct aromas and flavors to staple ingredients (*42*). Although

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

there are too many to name here, some notable examples of spices commonly used in Indian cooking include cumin, coriander, turmeric, mustard, ginger and clove. The diet is rounded out by a generous variety of fruits and vegetables, dairy products such as milk, yogurt, and fresh cheese, and condiments such as relishes, pickles and chutneys (*43*).

Indians typically eat two or three major meals throughout the day, supplemented by smaller meals or snacks, known as *tiffin* (*42*). Snacking is, therefore, a daily phenomenon for many Indians, and there are a variety of traditional snacks that they can choose from, including *samosas* (savory pastries), breads, and fried vegetables. In particular, there is a class of crunchy snacks, most made from some combination of cereals (largely rice) or legumes (chickpeas, green peas, and black or green lentils are common) (*44*). These base ingredients may be roasted or fried whole and eaten as is, or ground into flours to make doughs which can be formed into a variety of shapes and styles such as flat discs and long, noodle-like shapes. These dough products are usually deep-fried in hot oil, or are dried and fried just before consumption. The basic ingredients for these snacks are similar, so specific types are distinguished mainly by the shape and preparation method, as well as the spices used to season the snack. Some characteristic examples include *sev* (a thin fried noodle made from chickpea flour, cumin and pepper), *muri* (puffed rice), *papadum* (a flat wafer made of legume flour that is fried or roasted before consumption), and *murukku* (a coiled rope or ribbon-shaped snack typically made from black lentil flour, rice, cumin, and red chili powder) (*44*).

An interesting omission from the Indian diet is the soybean. Despite its prevalence in the diet of neighboring Asian countries such as China, India consumes only 0.22 kg of soybeans per capita annually (Table III). In fact, they are well below the world average of 1.72 kg as well. This is in spite of the fact that India is one of the top five soybean growing countries in the world, with an estimated 9.65 million metric tons produced during 2008-2009 (*45*). Trade data indicate that although soybeans are available, only the soybean oil is utilized domestically, and the defatted soy meal is exported or used for animal feed (*45*, *46*).

Since soybean and soy-related food products are unfamiliar to the Indian population, it is not surprising that some researchers have reported them as poorly accepted by Indian consumers (*47*). However, several studies have evaluated the acceptance of various soy food products by Indian subjects with favorable results (*16*, *23*, *47–49*). Neely (*29*) evaluated the acceptance of soy-based extruded snacks, and found them to be highly acceptable by both consumers familiar with Indian snacks but living in the United States, as well as consumers living in India, suggesting this type of product may be successful if marketed commercially, and could be one way to deliver soy to Indians. Perhaps, despite its unfamiliarity, the Indian population is more willing to accept soy foods than researchers may have initially hypothesized.

Table III. 2003 Annual per capita consumption of soybeans by region, Food and Agriculture Organization Food Balance Sheets

Country or region	*kg/year*
Korea	8.15
China	3.91
Myanmar	2.26
India	0.22
US	0.04
Asia	2.44
World	1.72

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Table IV. Winning and losing elements influencing consumer perception of food bars as determined by conjoint analysis. Use with permission

Silo	*Element*	*Utility Score*
Winning elements		
Calories	Low calorie (101-149 calories/bar)	11
Ingredients	Contains whole grain/oats	10
Ingredients	Contains chocolate	9
Calories	Light/diet (<100 calories/bar)	8
Macro-nutrients	High protein	7
Losing elements		
Calories	Medium calorie (150-249 calories/ba	-1
Healthy factor	Soy health claim	-2
Ingredients	Contains soy	-4
Type of bar	Meal replacement bar	-6
Calories	High calorie (>250 calories/bar)	-15

Concept Testing and Marketing Strategies for Extruded Soy Foods

Although soy foods in general are increasing in market share and popularity, it can be assumed that some categories are still not well accepted due to unfamiliarity or perceived poor sensory attributes (*50*). Amongst the extruded soy food varieties, breakfast cereals are a category of particular interest, as they are one of the categories showing strong market growth, while conversely, the salted soy snack category failed to increase market share in the US in 2007 (*51*). In order to best capitalize on the growth seen in some soy foods categories, a better understanding of consumer perceptions is necessary to guide more effective marketing strategies.

Conjoint analysis is a method to evaluate product concepts by examining trade-offs that consumers make when forming a judgment, thus indicating which attributes are most important (*52*). In a study by Lee and others (*53*), conjoint analysis was utilized to assess the effect of including soy ingredients on consumers' reactions to a healthy breakfast cereal. They found that although soy-related attributes contributed somewhat positively to almost half of the consumers' opinions of healthy breakfast cereals, that for the remaining consumers, soy attributes imparted negative connotations unless specific sensory attributes were also described. In a related follow-up study, two of the segments identified by conjoint analysis were further probed using focus group methodology (*54*). Here, participants expressed concerns about poor sensory characteristics of soy products, as well as confusion about the validity of soy-related health claims, while emphasizing the need for soy products to be affordable so as to encourage consumer purchase. These findings were echoed in another conjoint study on food bars, where consumer liking of the bar concepts suffered with the inclusion of soy-related attributes (*55*). Losing elements included the use of soy ingredients, as well as the presence of a soy health claim on the label (Table IV). As with healthy breakfast cereal, the authors also attributed the results to poor sensory properties of soy products coupled with ambiguous marketing messages about soy health effects. Therefore, it would seem that more efforts are necessary to assure and persuade consumers of the positive effects of soy foods consumption, particularly in the snack and cereal categories.

Concluding Remarks

Based on the works mentioned here, it appears that extruded soy snacks have the potential to deliver both exceptional health benefits and highly acceptable sensory properties. However, marketing techniques such as conjoint analysis seem to indicate that consumers are still wary of soy foods, so more efforts must be made to put consumers at ease and to communicate the truth to consumers. Carefully designed sensory evaluation methods can help elucidate the ways in which soy foods can be best designed to meet consumer preferences, particularly in the areas of the globe where soy foods potential has not yet been fully realized.

References

1. Messina, M. Modern applications for an ancient Bean: soybeans and the prevention and treatment of chronic disease. *J. Nutr.* **1995**, *125*, 567S–569.
2. The State of Food Insecurity in the World, 2004. Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/007/y5650e/y5650e00.htm (accessed June 1, 2009).
3. Bressani, R.; Elias, L. G. All-vegetable protein mixtures for human feeding. The development of INCAP Vegetable Mixture 14 based on soybean flour. *J. Food Sci.* **1966**, *31*, 626–631.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

4. Jansen, G. R.; Harper, J. M.; O'Deen, L. Nutritional evaluation of blended foods made with a low-cost extruder cooker. *J. Food Sci.* **1978**, *43*, 912–915.
5. Conway, H. F.; Anderson, R. A. Protein-fortified extruded food products. *Cereal Sci. Today* **1973**, *18*, 94–97.
6. Baskaran, V.; Bhattacharaya, S. Nutritional status of the protein of corn-soy based extruded products evaluated by rat bioassay. *Plant Food Hum. Nutr.* **2004**, *59*, 101–104.
7. Almeida-Dominguez, N. G.; Valencia, M. E.; Higuera-Ciapara, I. Formulation of corn-based snacks with high nutritive value: Biological and sensory evaluation. *J. Food Sci.* **1990**, *55*, 228–231.
8. Boonyasirikool, P.; Charunuch, C. Development of nutritious soy fortified snack by extrusion cooking. *Kasetsart J. (Natural Science)* **2000**, *34*, 355–365.
9. Che Man, Y. B.; Abdul Karim, M. N. B.; Kun, T. T. Evaluation of four high-protein rice-soy snack formulations. *Int. J. Food Sci. Technol.* **1992**, *27*, 715–719.
10. Siegel, A.; Lineback, D. R.; Bhumiratana, A. Development, acceptability, and proximate analyses of high-protein, rice-based snacks for Thai children. *J. Food Sci.* **1976**, *41*, 1184–1188.
11. Bakar, J.; Hin, Y. S. High-protein rice-soya breakfast cereal. *J. Food Process. Preserv.* **1985**, *8*, 163–174.
12. Bressani, R.; Braham, J. E.; Elías, L. G.; Cuevas, R.; Molina, M. R. Protein quality of a whole corn/whole soybean mixture processed by a simple extrusion cooker. *J. Food Sci.* **1978**, *43*, 1563–1565.
13. Mustakas, G.; Griffin, E.; Allen, L.; Smith, O. Production and nutritional evaluation of extrusion-cooked full-fat soybean flour. *J. Am. Oil Chem. Soc.* **1964**, *41*, 607–614.
14. Harper, J. M. *Extrusion of Foods*; CRC Press, Inc.: Boca Raton, FL, 1981; Vol. 2.
15. Park, K. Soybean Fortification of Extrusion Puffed Cereal Snack Foods. M.S. Thesis, University of Illinois at Urbana-Champaign, Urbana, IL, 1973.
16. Adesina, A. A.; Sowbhagya, C. M.; Bhattacharya, S.; Zakiuddin Ali, S. Maize-soy-based ready-to-eat extruded snack food. *J. Food Sci. Technol. Mys.* **1998**, *35*, 40–43.
17. Breen, M. D.; Seyam, A. A.; Banasik, O. J. The effect of mill by-products and soy protein on the physical characteristics of expanded snack foods. *Cereal Chem.* **1977**, *54*, 728–736.
18. Camire, M. E.; King, C. C. Protein and fiber supplementation effects on extruded cornmeal snack quality. *J. Food Sci.* **1991**, *56*, 760–763.
19. Martinez-Flores, H. E.; Cruz, M. C.; Larios, S. A.; Jiménez, G. E.; Figueroa, J. D. C.; Gómez-Aldapa, C. A. Sensorial and biological evaluation of an extruded product made from corn supplemented with soybean and safflower pastes. *Int. J. Food Sci. Technol.* **2005**, *40*, 517–524.
20. Wansink, B. Changing eating habits on the home front: Lost lessons from World War II research. *J. Public Policy Mark* **2002**, 90–99.
21. Lawless, H. T.; Heymann, H. *Sensory Evaluation of Food: Principles and Practices*; Aspen Publishers, Inc.: Gaithersburg, MD, 1999.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

22. Meilgaard, M.; Civille, G. V.; Carr, B. T. *Sensory Evaluation Techniques*; CRC Press, Inc.: Boca Raton, FL, 2006.
23. Senthil, A.; Ravi, R.; Bhat, K. K.; Seethalakshmi, M. K. Studies on the quality of fried snacks based on blends of wheat flour and soya flour. *Food Qual. Prefer.* **2002**, *13*, 267–273.
24. Baskaran, V.; Mahadevamma; Malleshi, N. G.; Shankara, R.; Lokesh, B. R. Acceptability of supplementary foods based on popped cereals and legumes suitable for rural mothers and children. *Plant Food Hum. Nutr.* **1999**, *53*, 237–247.
25. Boonyasirikool, P.; Charunuch, C. Development of nutritious soy flour snack food by extrusion cooking. *Food* **2000**, *30*, 177–196.
26. Obatolu Veronica, A.; Omueti Olusola, O.; Adebowale, E. A. Qualities of extruded puffed snacks from maize/soybean mixture. *J. Food Process. Eng.* **2006**, *29*, 149–161.
27. Faller, J. Y.; Klein, B. P.; Faller, J. F. Acceptability of extruded corn snacks as affected by inclusion of soy protein. *J. Food Sci.* **1999**, *64*, 185–188.
28. Yeu, K.; Lee, Y.; Lee, S.-Y. Consumer acceptance of an extruded soy-based high-protein breakfast cereal. *J. Food Sci.* **2008**, *73*, S20–S25.
29. Neely, E. Development and Cross-Cultural Consumer Acceptance of Indian-Style Soy Based Extruded Snack Foods. M.S. Thesis, University of Illinois at Urbana-Champaign, Urbana, IL, 2009.
30. Kandiah, J.; Laird, J. Sensory attributes and acceptance of flavored soy nuts by college students. *Nutr. Res.* **2002**, *22*, 405–410.
31. Simonne, A. H.; Weaver, D. B.; Wei, C. Immature soybean seeds as a vegetable or snack food: Acceptability by American consumers. *Innovative Food Sci. Emerging Technol.* **2000**, *1*, 289–296.
32. Faller, J. Y.; Klein, B. P.; Faller, J. F. Characterization of corn-soy breakfast cereals by generalized procrustes analyses. *Cereal Chem.* **1998**, *75*, 904–908.
33. Faller, J. F.; Faller, J. Y.; Klein, B. P. Physical and sensory characteristics of extruded corn/soy breakfast cereals. *J. Food Qual.* **2000**, *23*, 87–102.
34. Rhee, K. S.; Kim, E. S.; Kim, B. K.; Jung, B. M.; Rhee, K. C. Extrusion of minced catfish with corn and defatted soy flours for snack foods. *J. Food Process. Preserv.* **2004**, *28*, 288–301.
35. MacFie, H. Preference Mapping and Food Product Development. In *Consumer-Led Food Product Development*; MacFie, H., Ed.; CRC Press: Boca Raton, FL, 2007.
36. Thompson, J. L.; Drake, M. A.; Lopetcharat, K.; Yates, M. D. Preference mapping of commercial chocolate milks. *J. Food Sci.* **2004**, *69*, 406–413.
37. Young, N. D.; Drake, M.; Lopetcharat, K.; McDaniel, M. R. Preference mapping of cheddar cheese with varying maturity levels. *J. Dairy Sci.* **2004**, *87*, 11–19.
38. Guàrdia, M. D.; Guerrero, L.; Gelabert, J.; Gou, P.; Arnau, J. Sensory characterisation and consumer acceptability of small calibre fermented sausages with 50% substitution of NaCl by mixtures of KCl and potassium lactate. *Meat Sci.* **2008**, *80*, 1225–1230.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

39. Moskowitz, H. W.; Kumaraiah, V.; Sharma, K. N.; Jacobs, H. L.; Sharma, S. D. Cross-cultural differences in simple taste preferences. *Science* **1975**, *190*, 1217–1218.
40. Rozin, P. How Does Culture Affect Choice of Foods? In *Consumer-Led Food Product Development*; MacFie, H., Ed.; CRC Press: Boca Raton, FL, 2007; pp 66−80.
41. Achaya, K. T. *Indian Food: A Historical Companion*; Oxford University Press: Delhi, India, 1994.
42. Sen, C. T. *Food Culture in India*; Greenwood Press: Westport, CT, 2004.
43. Hughes, M.; Mookherjee, S.; Delacy, R. *World Food India*; Lonely Planet Publications: Footscray, Australia, 2001.
44. Mudambi, S. R.; Rajagopal, M. V. Snack Foods in India. In *Snack Foods Processing*; Lusas, E. W., Rooney, L. W., Eds.; Technomic Publishing Co.: Lancaster, PA, 2001.
45. Soybeans: World Supply and Distribution. United States Department of Agriculture Foreign Agriculture Service. http://www.fas.usda.gov/psdonline/psdReport.aspx?hidReportRetrievalName=Table+07%3a+Soybeans%3a+World+Supply+and+Distribution&hidReportRetrievalID=706&hidReportRetrievalTemplateID=8 (accessed June 1, 2009).
46. Gandhi, A. P.; Kotwaliwale, N.; Taimini, V.; Kawalkar, J.; Srivastav, D. C.; Parihar, V. S.; Raghu Nadh, P. Effect of incorporation of defatted soyflour on the texture of chapatis. *J. Texture Stud.* **2000**, *31*, 609–614.
47. Jayalakshmi, N.; Neelakantan, S. Studies on the acceptability of sorghum-Soya blends in south Indian dishes and their keeping quality. *Indian J. Nutr. Diet* **1987**, *24*, 136–141.
48. Gandhi, A. P.; Mishra, V. K.; Ali, N. Organoleptic assessment of full fat soy flour in various indigenous products. *J. Food Technol.* **1983**, *18*, 771–775.
49. Deshpande, S. S.; Joshi, K. C.; Bargale, P. C.; Jha, K.; Singh, V.; Varghese, S. Development, acceptability and shelf-life studies of soy-fortified maize sattu. *J. Food Sci. Technol. Mys.* **2004**, *41*, 674–678.
50. Wansink, B.; Cheong, J. Taste profiles that correlate with soy consumption in developing countries. *Pak. J. Nutr.* **2002**, *1*, 276–278.
51. Lummis, D. *Soy Foods and Beverages in the U.S.*; Packaged Facts: Rockville, MD, 2008.
52. Wittink, D. R.; Cattin, P. Commercial use of conjoint analysis: An update. *J. Mark.* **1989**, *53*, 91–96.
53. Lee, C. M.; Moskowitz, H. R.; Lee, S.-Y. Expectations, needs and segmentation of healthy breakfast cereal consumers. *J. Sens. Stud.* **2007**, *22*, 587–607.
54. Lee, C. M.; Lee, S.-Y. Consumer insights on healthy breakfast cereal: A focus group research. *J. Sens. Stud.* **2007**, *22*, 417.
55. Mahanna, K.; Moskowitz, H. R.; Lee, S.-Y. Assessing consumer expectations for food bars by conjoint analysis. *J. Sens. Stud.*, in preparation.

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch020

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Chapter 21

Flavor-Soy Protein Interactions

Inthawoot Suppavorasatit and Keith R. Cadwallader[*]

**Department of Food Science and Human Nutrition, University of Illinois,
1302 West Pennsylvania Avenue, Urbana, IL 61801
[*]cadwlldr@uiuc.edu**

Soy protein present in a food can interact with flavor compounds and result in the reduction of flavor intensity (flavor fade). This chapter provides an overview of recent studies on the binding of volatile flavor compounds to food proteins, specifically to soy proteins in both solid state and in aqueous model systems. The occurrence of flavor binding and techniques used to measure flavor-protein interactions are described. In addition, binding capacities of flavor compounds to different food proteins are compared and the factors affecting flavor-protein binding are discussed.

Flavor quality greatly affects the consumer acceptance of food and is, therefore, considered a major factor determining the commercial success of a newly launched food product (*1*, *2*). In the meantime, the demand for low-calorie healthy foods containing high protein and reduced fat, sugar and sodium is on the rise (*1*, *3*). Unfortunately, these “healthy” foods often do not satisfy the consumer due to their inferior flavors. In particular, although high protein foods are a popular choice, the addition of protein to a food product may alter its flavor either by imparting undesirable off-flavors or by changing the food’s flavor release/flavor perception profile due to flavor-protein binding interactions (*4*).

Soy protein is a popular food ingredient because of its functionality and potential health benefits. However, despite these benefits the consumption of soy foods in the United States and Europe is limited due to the presence of undesirable “green”, “beany” and “grassy” off-flavors (*5*). Furthermore, any flavor compounds added to a food product may interact with soy protein and result in flavor fade. To help solve these problems it is necessary to fully

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

understand the mechanisms involved in flavor-soy protein binding. In addition, this knowledge can be used to develop methods or processes to counteract the effects of flavor-protein interactions leading to more acceptable high protein food products (*6*).

Binding of Flavor Compounds by Food Proteins

In food systems, interactions between flavor (aroma) compounds and food matrix components (e.g., lipids, carbohydrates and proteins) can affect flavor perception. One critical factor that influences flavor perception is the flavor release rate. Lipids in food have the greatest impact on flavor perception since they can act as solvents for lipophilic flavor molecules and thus reduce the rate of flavor release during food consumption (*7*). Carbohydrates can bind to flavor compounds, especially polar molecules, via dipole-dipole interactions and hydrogen bonds. Thus, carbohydrates can affect flavor release and perception (*1*, *2*). Beside lipids and carbohydrates, proteins can also influence flavor perception. In particular the binding interaction of flavor molecules with protein can be especially problematic in protein-enriched foods leading to reduction or loss of flavor (flavor fade) and hence a decline in product acceptability (*8*).

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

General Flavor-Protein Interactions

Protein on its own does not impart much flavor, but it can alter flavor perception by binding with flavor compounds. Protein can bind with off-flavors or bind selectively with desirable flavors, and hence change the flavor profile of a food. When flavor compounds are added to food products containing proteins, the retention of flavors during processing and storage will be altered, thus making it difficult for food manufacturers to choose and control the proper level of flavoring necessary to achieve the desired flavor intensity in final product (*6*, *9*, *10*).

Changing the amino acid sequence can alter the chemical characteristics of a protein for several reasons. The binding properties of a protein are strongly influenced by its three-dimensional structure, which is formed as a result of disulfide bridges and hydrogen bonds between amino acids (*1*). Many studies have attempted to relate binding to the molecular structures of flavor compounds and proteins, but the results have been inconsistent due to the differences among proteins, flavor compounds and experimental conditions (*8*). In general, the type of interaction depends on the nature of proteins and flavor compounds and can be reversible (physicochemical) or irreversible (chemical) (*8*, *11*). Reversible binding includes hydrogen bonding, hydrophobic interactions and ionic bonds. In contrast, irreversible binding occurs when flavor compounds, especially aldehydes (such as vanillin) form covalent bonds (Schiff-bases) with the amide side chains of proteins (*8*, *12–14*). Gremli (*9*) suggested that reversible interactions may not necessarily be negative, in that this type of binding might protect flavor compounds during food processing so that they can be later released from the food during consumption.

Most studies related to flavor-protein interactions have been conducted with milk proteins (*14–35*). Other proteins studied include soy protein (*4*, *6*, *23*, *36–45*), fababean protein (*46*, *47*), ovalbumin (*48*, *49*), myofibrillar protein (*50*), broad bean protein (*51–53*) and sacroplasmic protein (myoglobin) (*54*).

Binding of Flavor to Soy Proteins

Similar to other proteins, soy protein can bind with flavor compounds in foods and cause flavor problems, resulting in a decrease in product acceptability. An understanding of this interaction and mechanisms that govern it can help to alleviate this problem through the development of better processing methods or alternative flavoring strategies (*6*). With respect to flavor-soy protein interactions, useful information has been obtained from the study of both solid state (low-moisture) and aqueous model systems.

Binding of Flavor Compounds to Soy Proteins in the Solid State

Soy proteins are important ingredients in many low-moisture food products such as snack bars, baked goods and cereal-based products. Understanding flavor-protein interaction in low-moisture foods is particularly important because low-moisture foods have a long shelf-life, and thus even slow reactions can lead to a decline in product quality. During storage, the flavor components of low-moisture foods can migrate into and out of the product leading to adsorption (either desirable or undesirable depending on the characteristics of the flavor compound) and a reduction in flavor intensity (flavor fade) (*55*). The relative humidity (RH) level is an important factor affecting the shelf-life of low-moisture food products. In addition, when the moisture migrates into and out of the food product, it can change the flavor retention/release properties of the food system (*56*). Consequently, to help control and maintain the desirable flavor of low-moisture products during storage, it is necessary to maintain the appropriate storage conditions.

Aspelund and Wilson (*6*) used thermodynamics as a tool to study the adsorption of selected off-flavor compounds, including homologous series of alcohols, aldehydes, ketones, hydrocarbons and methyl esters, onto dry soy protein isolate (SPI). They found that hydrocarbons were bound most weakly and alcohols most strongly. Their results demonstrated that the functional group of a flavor compound plays an important role on its binding to soy protein under dry conditions. They also found that the binding of flavor compounds to soy protein is driven by the enthalpy of adsorption in the gaseous system. Furthermore, it was hypothesized that the binding of SPI with flavor compounds occurs by the combination of nonspecific van der Waals forces and hydrogen bonding (*5*). The effect of processing parameters (temperature and moisture content) on the binding of off-flavor compounds with soy protein was studied by Crowther and others (*57*). These researchers determined the heat of adsorption and adsorption coefficients for the binding of alcohols, aldehydes and ketones to soy protein. They found that moisture and temperature affected the binding of flavor compounds due to protein denaturation.

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Recently, Zhou and Cadwallader developed an inverse gas chromatography (IGC) technique to study flavor-soy protein binding interaction under controlled relative humidity. They found that increasing RH from 0 to 30% caused a reduction in binding between flavor compounds (1-hexanol and hexanal) and SPI due to competition between water and the flavor molecules at the binding sites on the protein surface (*42*). In agreement with results of Aspelund and Wilson (*6*), they also found that the chemical structure of a flavor compound greatly affects binding. For non-polar flavor compounds (hydrocarbons), the main binding forces were nonspecific van der Waals dispersion forces, which were not affected by adsorbed water. On the other hand, both specific (hydrogen bonding and dipole forces) and nonspecific interactions were involved in the binding of more polar flavor compounds, including esters, ketones, aldehydes and alcohols. Also, they found that binding of these flavor compounds was weakened when water was adsorbed onto the dehydrated SPI (*43*). Evaluation of the binding of selected butter flavor compounds to soy proteins was investigated in a wheat soda cracker system (*44*). There was no effect on the binding of diacetyl and hexanal to the crackers due to the presence of soy proteins, but binding of γ-butyrolactone and butyric acid were strongly affected. These researchers suggested that the stronger binding observed for the soy cracker might be due to the greater polarity of this matrix (*44*). Furthermore, the competitive binding of selected volatile compounds (hexanol, hexanal, hexane and (*E*)-2 hexenal) by dehydrated SPI under controlled RH using IGC coupled with atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) was studied (*45*). The results showed strong competition between unsaturated and saturated aldehydes with respect to their binding interactions with SPI when alcohols were present. In contrast, there was no significant effect on binding of alcohols with SPI when in the presence of aldehydes or alkanes. In addition, alkanes did not influence the binding affinity of alcohols or aldehydes (*45*).

Binding of Flavor Compounds to Soy Proteins in Aqueous Model Systems

Flavor changes due to binding are known to occur under ambient conditions in aqueous media containing soy, for example, in soymilk during storage. These changes may be caused by release of previously bound "beany" flavor compounds of the soy protein itself, leading to off-flavors; or by binding interactions between the protein and added flavorings, thus causing flavor fade (*9*). Researchers have investigated the binding interactions of flavor compounds and soy proteins in aqueous model systems (*9*, *23*, *32*, *36–41*, *58*). The interaction of flavor compounds with soy protein in an aqueous system was initially studied using static headspace-gas chromatography (GC) (*9*). It was reported that alcohols underwent weak interactions with the soy protein, while unsaturated aldehydes, and to a lesser extent saturated aldehydes, strongly interacted. It was concluded that both reversible and irreversible binding interactions were involved (*9*).

Damodaran and Kinsella (*37*) used an equilibrium dialysis method to study the binding interaction of ketones (2-heptanone, 2-octanone, 2-nonanone and 5-nonanone) and nonanal with soy proteins in an aqueous model system. They

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

found that the binding constants increased with an increase in the chain length of the flavor molecule. The hydrophobic free energy of binding also decreased when the position of carbonyl group was shifted from the terminal end to the interior of the flavor molecule. They also concluded that the binding interaction of carbonyls with soy protein was relatively weak under aqueous conditions. Further studies by Damodaran and Kinsella (*36*) focused on the effect of the conformation of soy proteins on their flavor binding properties. Glycinin (11S) and β-conglycinin (7S) protein fractions were found to bind differently with 2-nonanone. Glycinin had almost no binding affinity to 2-nonanone, while β-conglycinin did not differ from whole soy protein. In addition, presence of urea or chemical modification (succinylation) of the protein also decreased the binding affinity of the protein to 2-nonanone. These results were in agreement with those of O'Neill and Kinsella (*38*), who demonstrated that the binding affinity of β-conglycinin was around five-fold greater than glycinin. However, these results contradicted those of O'Keefe and others (*40*), who investigated the influence of temperature on the binding properties of flavor compounds to soy proteins. These researchers found that the binding affinities of various flavor compounds, including aldehydes (butanal, pentanal, hexanal and octanal), ketones (2-hexanone, 3- hexanone, 2-nonanone and 5-nonanone), hexanol and hexane, were much greater for glycinin than for β-conglycinin. They also found that for aldehydes an increase in chain length caused an increase in their affinity to glycinin, while it had no effect relative to β-conglycinin binding. In 2002, Zhou and others (*41*) performed a comparison study of the binding affinities of β-conglycinin, glycinin and SPI with 2-pentyl pyridine (2PP). Their results showed that the binding affinity of 2PP to glycinin was the greatest, followed by β-conglycinin and SPI. Binding was greater under alkaline conditions than under neutral or acidic conditions. Greater binding of 2PP occurred at high temperature (74 °C) than at lower temperature (4 or 25 °C). This might be because of thermal denaturation of the protein, which can increase the ability of proteins to bind with 2PP (*41*). In contrast, the binding affinity at 4 °C was higher than at 25 °C, which agrees with the results of a previous studied by Damodaran and Kinsella (*37*) in which they demonstrated that protein hydrophobicity was greater at 5 °C than at 20 °C. The binding of alcohols by soy protein in aqueous solutions was studied using equilibrium dialysis method (*58*). These researchers found that the binding of alcohols to soy protein might involve hydrophobic interaction and hydrogen bonding. They also concluded that increasing in level of denaturation by heat treatment affected alcohol-protein binding by limiting hydrogen bond formation (*58*).

A comparison study of the binding of selected flavor compounds, such as vanillin, to soy and dairy proteins (casein and whey protein) was conducted by Li and others (*23*). They found that whey protein demonstrated the strongest binding affinity towards vanillin. Moreover, the binding of vanillin to dairy proteins was driven by the enthalpy, which might be due to the interaction of the carbonyl and hydroxyl groups of vanillin with the proteins. On the other hand, the binding of vanillin to soy protein was driven by entropy, which means that the conformation of the protein might influence the binding. Therefore, any parameter that could affect the conformation of soy protein, such as denaturation by heating, could also influence the binding of lactones (γ-9, γ-10, δ-10 and δ-11) with SPI, amino acids or

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

casein. The results showed that there was no difference in the degree of binding for the lactones on SPI. Study of the competitive binding of two lactones with similar structures, δ-10 and γ-11, to soy protein showed that there was some competition between these two lactones for the available binding sites on the protein molecule (*32*).

Techniques for Measuring Flavor-Protein Interactions

The molecular study of the interactions between flavor compounds and macromolecules, such as proteins has been conducted by different approaches, including instrumental techniques and sensory analyses (*8*). Instrument techniques have been used as a popular option for studying flavor-protein interaction for decades. However, instrumental results do not directly relate to consumer perception of flavor in a real food system. Therefore, sensory analyses are necessary to correlate or relate instrumental measurements to consumer acceptance data (*59*).

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Conventional Techniques

The two main methods commonly used to study flavor-protein interactions are static headspace-gas chromatography (SH-GC) and equilibrium dialysis (*7*, *36*, *37*, *39*, *40*). Both methods are conducted under equilibrium conditions and the systems are often considered to be simple, since they are limited to the study of the binding of a single flavor compound to a single protein (*8*).

SH-GC techniques are based on measurement of the vapor-liquid partition equilibrium in a well-defined system (*8*). These methods measure the change in the partition coefficient by directly determining the change in volatile concentration in the headspace above the food or model system at equilibrium (*7*). In a SH-GC, a known amount of flavor compound is added to a buffered protein solution, the mixture is allowed to reach equilibrium, and then the volatile concentration in the headspace is measured by GC. The difference between the volatile concentration above the protein solution and the blank buffer solution (control) is then compared (*7*, *39*, *40*). This technique provides a simple and straightforward means to measure the impact of flavor-protein interactions, especially in liquid products. However, headspace techniques are unsuitable for semi-volatile compounds. In this case, a large amount of sample is needed for adequate detection. In addition, this technique is also limited since it does not provide kinetic information, thus making it difficult define which binding mechanisms are involved (*7*, *8*). To resolve the problem of poor sensitivity, splitless or on-column GC techniques can be used. Furthermore, increasing the equilibrium temperature can help, but thermal reactions may occur (*8*).

A popular alternate to increase the sensitivity and utility of the headspace technique has been application of headspace solid-phase microextraction (SPME) (*25*, *34*, *48*, *54*, *60*). It was found that SPME is good for both static and dynamic headspace analysis for measurement of milk protein-flavor interactions (*25*). In addition, SPME can also be used to measure the flavor concentration using an equilibrium dialysis technique (*41*). Pawliszyn and coworkers developed SPME around 1990 by using fused silica fiber coated with thin layer of a selective coating to adsorb (or absorb) the volatile flavor compounds from the headspace above the sample. Then the adsorbed (or absorbed) volatile flavor compounds can be analyzed by thermally desorption into the GC for analysis (*60–62*). SPME is a sensitive, rapid, inexpensive, selective and solvent-free sampling technique, and is suitable for automation. In addition, it can be used with many separation methods including GC, GC-MS and high performance liquid chromatography (HPLC) (*63*). However, SPME is difficult to use with external standards in some complex matrices and the bias in quantitative analysis can be caused by the competition of flavor compounds for the fiber. Therefore, it can be concluded that SPME is more suitable for simple systems than complex ones (*8*, *64–66*).

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Dialysis is another technique that has been commonly used for studying flavor-matrix binding under equilibrium conditions (*7*, *8*). This method is based on liquid-liquid partition equilibrium. The ligands (flavor compounds), which are not bound with the specific food component (such as protein) at equilibrium, are measured. In a dialysis cell system, the protein solution and the solution containing the flavor compound of interest are separated by a semi-permeable membrane in a twin chambered cell. Then the cell is shaken at constant temperature until equilibrium is reached. The flavor compounds in the solutions are extracted and the concentrations are determined by GC (*7*, *36*, *37*). For a flavor compound with low volatility, such as vanillin or benzaldehyde, HPLC or UV-VIS methods can be used to determine free (unbound) flavor compound after equilibrium with the protein (*8*, *19*, *23*, *26*, *46*).

As described above, equilibrium dialysis can be widely used for the study of flavor-protein interaction. It can provide useful information including equilibrium binding constant, number of binding sites and useful thermodynamic parameters can be calculated to show the nature of binding. However, this method is very time consuming and the flavor compounds might be lost during testing (*8*). There are some other factors that can alter the binding including pH, reducing agents contained in the buffer, solvent extraction procedures and the dialysis membrane might be plugged or bind with ligands (*5*). Furthermore, this method is not suitable for the study of solid (dry) systems.

Inverse Gas Chromatography Technique

Inverse gas chromatography (IGC) has been widely used for the characterization of surface physicochemical properties of solid substances based on gas-solid adsorption chromatography theory (*67*, *68*). It has been applied for the characterization of the surface of polymers and their interactions with fragrance molecules (*69*). In contrast to conventional GC, the roles of the stationary phase and mobile phase are inverted. In IGC, the subject of interest is the non-volatile substance. The GC column is prepared by packing the non-volatile substance (stationary phase), then injecting known amounts of volatile compounds (volatile probes), which are have known structures and physical properties, into the GC. The surface chemistry, such as surface sorption and phase transition, of the non-volatile solid substance (stationary phase) can be obtained by IGC based on the partitioning of the volatile probes between the mobile and stationary phases. In addition, the thermodynamic properties of the sorbate-sorbent system can be measured at the same time (*42*, *67*, *68*).

IGC has been mostly used in the material science and chemical engineering fields. However, IGC has been applied in the food science discipline, mainly for the determination of water sorption isotherms for dry foods (*70*). IGC technique can be used as a tool to study binding properties of small ligands (flavor compounds) to food substances (such as proteins) under dry (moisture free) or low moisture conditions (*4*, *6*, *42–45*). There are some advantages of IGC over the conventional methods including its simplicity, speed and accuracy. In addition, it is suitable for the study of dry and semidry food materials (*42*).

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Sensory Analysis

Instrumental analysis of flavor-protein interactions provides useful information on the mechanisms involved, which is important for understanding the nature of the binding. However, the results obtained from a model system may not be directly applicable to real foods, since the instrumental technique cannot give the actual impact of flavor-protein interaction when the food is consumed. The use of sensory analysis can provide additional information related to the effect of binding on flavor perception and product acceptability. Correlation of the instrumental and sensory data can then provide a better understanding of the cause-and-effect relationship of the flavor-protein interactions. The knowledge obtained from the combined studies can help food producers develop improved food formulation with more acceptable flavors (*8*, *44*). However, to obtain precise sensory results, intensive training of the panelists is often necessary. Also, sensory analysis can be expensive and time consuming (*8*).

One of the methods used for sensory study is sniffing (odor evaluation), which is the perception of aroma intensity of volatile flavor compounds present in the gas phase (headspace) above the food. The amount of flavor compounds in the headspace is determined by the distribution the volatile components between the headspace and food matrix, which is affected by flavor-matrix interactions (*71*, *72*). Zhou and others (*44*) evaluated flavor binding of selected volatile butter flavor compounds (diacetyl and butyric acid) onto soy containing crackers using IGC and sensory techniques. They found the general agreement between the IGC and sensory evaluation data. Other studies have examined the effect of flavor-protein interactions on flavor perception (*19*, *47*, *73–75*). Ng and others (*47*) compared the sensory perception of vanillin versus free vanillin measured instrumentally (HPLC) in a fababean protein model system. They showed that free vanillin contributed to perceived flavor, and concluded that it is possible to use instrumental results to predict human perception of specific flavor compounds in a food system containing both flavoring and protein. Hansen and Heinis (*73*) using flavor perception studies found that vanillin flavor intensity declined in solutions containing either sodium caseinate or whey protein concentrate. Similar to vanillin, *d*-limonene intensity was also decreased in the present of proteins in the solution. For benzaldehyde, the flavor intensity declined only in the presence of whey protein concentrate (*74*). Another study concerned with the effect of heat and emulsifier addition on the interaction of vanillin with milk proteins made use of sensory and HPLC techniques. A reduction of flavor perception was observed, but there was no correlation between sensory and HPLC results (*19*).

Other Techniques

The aforementioned equilibrium methods are suitable for the study molecular interactions between volatile compounds and proteins or other ingredients in food matrices. However, they do not provide enough information about the nature of these interactions. Spectroscopic techniques can be used to obtain more information about the nature of the interactions by providing conformational changes while proteins are modified (*8*).

Fluorescence spectroscopy is one of the tools for the investigation of the structure, function and reactivity of biological molecules, including proteins. This technique is fast and simple. In addition, as compared to light absorption techniques, fluorescence spectroscopy has 100 to 1000X greater sensitivity. Information about the local interactions can be acquired by investigation of wavelength shifts and fluorescence emission intensity of tryptophan residues in the proteins. For example, when the binding between flavor compounds and protein molecule occurs the conformation of the protein itself might change. Binding constants and also number of binding site can be determined in terms of the change in fluorescence intensity (*18*, *24*, *31*). However, environmental effects (such as pH) can interfere with the optical effects and lead to inaccurate results. Also, the protein studied must contain at least one tryptophan residue (*76*).

Nuclear magnetic resonance (NMR) spectroscopy is a suitable technique for the study of conformation changes at the atomic level. It is a useful technique to investigate intra- and intermolecular interactions. NMR spectroscopy has been used for the study of the conformation changes of milk protein as affected by temperature, pH and high pressure treatments (*77*, *78*). Therefore, NMR spectroscopy is potentially very useful for the study of mechanisms involved in protein-flavor interactions. Lübke and others (*27*) studied the conformation changes of β-lactoglobulin caused by the binding of flavor compounds. They found that the binding mechanisms were disclosed by using two-dimensional (2D) NMR technique. NMR data provided precise information of binding location and confirmed findings from previous studies, which were done by using fluorimetry, affinity chromatography and infrared spectroscopy methods. Furthermore, diffusion-based NMR techniques, which are fast and easy to perform, were proposed as rapid screening techniques in the study of molecular interactions between flavor compounds and biological macromolecules, but the methods lack sensitivity. The diffusion-based nuclear Overhauser effect (NOE) pumping method, which is the combination of pulsed field gradient nuclear magnetic resonance spectroscopy (PFG-NMR) and a NOE experiment, can be used to screen and identify which flavor compounds in the mixture are selectively bound to proteins. Diffusion-based NMR methods can be more precise if the experiments are combined with 2D-NMR techniques to give more information about specific binding sites and also mechanisms involved (*27*, *79*).

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Fourier transform infrared (FT-IR) spectroscopy is another technique that has been used to investigate changes in the secondary and tertiary structures of biological macromolecules. It was recently used for the study of the aggregation and structural properties of SPI as affected by high pressure treatment (*80*). Along with NMR, FT-IR spectroscopy has been used to discriminate or screen aroma compounds. The discrimination is based on the protein spectral changes in the amide I band (1700-1600 cm^{-1}), which is from the amide bonds that link the amino acids and the secondary structure of proteins. When FT-IR results are combined with the specific binding site information (for example, strand β-G, α helix and strand β-I) obtained from NMR techniques, the relationship between the ligand structure and their binding behaviors can be obtained (*35*).

Determination of Binding Parameters

Characterization of the parameters involved in flavor-protein binding is important for the understanding of the mechanisms involved. The most frequently used technique assumes that there is equilibrium between a protein molecule (*P*) and single ligand (*L*). This simplest case can be described as follows (*7*, *81*, *82*):

$$P + L \leftrightarrows PL$$

The equilibrium binding constant, *K*, for this reaction is defined by:

$$K = \frac{[PL]}{[P][L]} \quad \text{or} \quad [PL] = K[P][L]$$

If $[P(total)] = [PL] + [P]$, therefore $[PL] = K[L]([P(total)] - [PL])$, and

$$\frac{[PL]}{[P(total)]} = \frac{K[L]}{1 + K[L]}$$

Since υ is the number of moles of ligand bound per mole of total protein and is equal to $\frac{[PL]}{[P(total)]}$, then:

$$\upsilon = \frac{K[L]}{1 + K[L]}$$

If one type of ligand can bind to more than one site on a protein (*n* binding sites), the equation will be *n* times that of one binding site, with the same equilibrium binding constant, *K*, therefore:

$$\upsilon = \frac{nK[L]}{1 + K[L]} \quad \text{or} \quad \frac{\upsilon}{[L]} = Kn - K\upsilon$$

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Table I. Equations and parameters commonly used in ligand-protein binding studies

Equation	*Plot*	*Binding constant (K)*	*Number of binding sites (n)*
Scatchard plot			
$\frac{\upsilon}{[L]} = Kn - K\upsilon$	$\frac{\upsilon}{[L]}$ vs υ	Slope = $-K$	y-intercept = nK
Klotz plot			
$\frac{1}{\upsilon} = \frac{1}{n} + \frac{1}{Kn[L]}$	$\frac{1}{\upsilon}$ vs $\frac{1}{[L]}$	Slope = $\frac{1}{Kn}$	y-intercept = $\frac{1}{n}$

This equation can be rearranged into a Scatchard plot, which is the plot between $\frac{\upsilon}{[L]}$ vs υ. The slope of the plot will give the value of $-K$, and y-intercept gives the value of nK. In addition, the above equation can be rearranged to a form most commonly used in ligand-protein binding studies (Scatchard equation) (*7*, *83*):

$$\frac{1}{\upsilon} = \frac{1}{n} + \frac{1}{Kn[L]}$$

The plot between $\frac{1}{\upsilon}$ vs $\frac{1}{[L]}$ gives a double reciprocal plot, or Klotz plot (*84*, *85*). The slope of the plot gives the value of $\frac{1}{Kn}$, while the y-intercept gives the value of $\frac{1}{n}$. The equation parameters for both Scatchard and Klotz plots are summarized in Table I.

Since the measurement is conducted at constant temperature, the value of the binding constant can be used to determine the thermodynamic parameters that relate to binding including free energy of binding, enthalpy of binding and entropy of binding as follows (*7*):

The free energy of binding (ΔG)

$$\Delta G = -RT\ln K$$

The enthalpy of binding (ΔH)

$$\Delta H = \frac{-Rd\ln K}{d(1/T)} \text{ and}$$

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

The entropy of binding (ΔS)

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

As mentioned above, Scatchard and Klotz plots are the models usually used. The assumption of both models is that the protein must have equal and independent binding sites. If the binding sites in protein are not equal and dependent, they can cause either the Scatchard or Klotz plots to be non-linear. Another plot that can be used in when these two plots are non-linear is a Hill plot. The Hill equation is as follow:

$$\upsilon = \frac{n}{\dfrac{1}{(K[L])^h} + 1}$$

Where h is the Hill coefficient, which reflects the cooperation between binding sites. The Hill equation can be rewritten in double-reciprocal form as follows:

$$\frac{1}{\upsilon} = \frac{1}{n(K[L])^h} + \frac{1}{n}$$

Similar to the Klotz plot, the Hill plot is the plot between $\frac{1}{\upsilon}$ vs $\frac{1}{[L]}$ (*8*, *86*, *87*).

Besides the thermodynamic parameters, protein hydrophobicity is also considered in many flavor-protein binding studies. When protein is unfolded, the non-polar groups in protein will be exposed to the environment. These non-polar groups are responsible for the hydrophobic binding of proteins with other ligands (*39*, *40*). Also, there might be some correlation between hydrophobicity and either the entropy or enthalpy of binding. In the case of the headspace technique, the concentration in the headspace is one of the parameters that is considered (*50*). In addition, the partition coefficient, which is the ratio of the concentration of volatile compound in gas phase to its concentration in liquid phase, can also be calculated (*60*, *88*).

Comparison of Flavor Binding Capacities of Soy Proteins with Other Food Proteins

Selected binding parameters determined by different research groups for selected flavor compounds (aldehydes and ketones) with various proteins (such as soy proteins and dairy proteins) are shown in Table II. The techniques used in these studies differed, which might explain why there are differences among the results for even the same protein type, flavor compound and experimental conditions.

Carbonyl compounds (ketones and aldehydes) are the flavor compounds most often chosen for study. Researchers tended to use the same flavor compounds to study flavor binding properties of different proteins. For example, the Kinsella research group used 2-nonanone as a model flavor compound to compare binding properties of whole soy protein, SPI, soy protein fractions (7S and 11S) and β-lactoglobulin (β-lg). They selected equilibrium dialysis as a tool for their study, which makes it possible to compare the binding parameters determined in each study (*16*, *37*, *38*). Other researchers also used 2-nonanone in to study other types of proteins including whey protein isolate, whey protein concentrate (WPC), β-lg and bovine serum albumin (BSA) (*31*, *33*, *78*); however, those results cannot be readily compared because different techniques and experimental condition were employed by the various research groups.

To compare binding capacities of soy proteins with other food proteins, results for the same flavor compounds, same techniques and same experimental conditions should only be considered. Parameters for the binding interaction of 2-nonanone with various proteins based on the equilibrium dialysis technique are compared in Table II. Only a slight difference exists for the number of binding sites (n) and the binding constants (K) among whole soy protein (5.5 and 570 M^{-1}), soy protein isolate (SPI) (4 and 930 M^{-1}) and glycinin (11S) fraction (3.1 and 540 M^{-1}). However, n and K differed between β-conglycinin (7S) fraction (1.8 and 3050 M^{-1}) and the aforementioned proteins (*37*, *38*). It is therefore concluded that β-conglycinin has higher affinity for 2-nonanone than does whole soy protein, SPI or glycinin.

Based on results of O'Neill and Kinsella (*16*), the binding constant of β-lg with 2-nonanone is 2440 M^{-1}, which is greater that what was observed for soy protein and its fractions. When comparing binding affinities, the negative free energy of binding (ΔG) should also be considered. The negative ΔG values for β-lg and SPI were 4620 and 4045 cal/mol, respectively. Based on these values it can be concluded that β-lg has a greater affinity for 2-nonanone than does SPI. The data in Table II should be interpreted cautiously, since they differ from results of other researcher groups who used different methods. For example, Liu and others (*31*), who used a headspace dialysis technique, reported values that were over 20000-fold higher than what was reported by O'Neill and Kinsella (*16*) even though the same protein was studied. Therefore, it can be concluded that data generated from different techniques should not be directly compared.

Another flavor compound that is of great interest is vanillin. This popular flavor compound is commonly added into soy-based beverages. Li and others (*23*) compared the binding of vanillin to three types of protein, including SPI, sodium caseinate and WPI. They found that the number of binding sites for SPI was higher than for the other two proteins which contained about the same number of binding sites. The binding constant for WPI was 1713 M^{-1}, which was higher than SPI (683.5 M^{-1}) and sodium caseinate (352.7 M^{-1}). The negative free energy of binding (ΔG) for WPI was highest, followed by SPI and sodium caseinate (4217, 3696 and 3322 cal/mol, respectively). From both binding constants and ΔGs, it was indicated that the affinity of WPI for vanillin was higher than those of SPI and sodium caseinate. In addition, these data can be compared with those Burova and others (*89*), who studied the affinity of BSA for vanillin using an equilibrium dialysis technique. Number of binding sites for BSA was 2, which was not much different from other proteins, while the dissociation constant for

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

BSA was much higher than what was reported for WPI, SPI and sodium caseinate. Based on these findings, BSA appears to have a higher affinity towards vanillin as compared with the above three proteins. However, there were no data on ΔG and the temperature used in the study was 298K (13K higher). Moreover, the data obtained (shown in Table II) cannot be compared with those of Mikheeva and others (*90*) who investigated the binding of vanillin with β-lg, BSA and ovalbumin because that research group used a UV-VIS method in their study. In addition, the study from Li and others (*23*) reported changes in enthalpy (ΔH) and entropy (ΔS), which are not shown in Table II. They concluded that the interaction of vanillin with sodium caseinate and WPI was driven by enthalpy because their ΔH and ΔS values were negative (-1264 cal/mol and -32.70 cal/K•mol, respectively, for sodium caseinate and -8495.76 cal/mol and -15.01 cal/K•mol, respectively for WPI). In contrast, interaction of vanillin with SPI was driven by entropy due to the highly positive in enthalpy (7424 cal/mol), which was endothermic. The binding, which happened naturally due to the entropy change, was high (39.02 cal/K.mol), which also resulted in a negative ΔG. This result is in good agreement with the work from Aspelund and Wilson (*6*) who also found entropy drove the interaction of SPI with hexanal and hexanone. Therefore, it can be concluded that a change in conformation of SPI due to protein unfolding, and which is confirmed by a high entropy value, plays a key role in the interaction of soy protein with vanillin.

Table II. Binding parameters for selected flavor compounds (aldehydes and ketones) with various food proteins

Protein	*Flavor compound*	n^a	K^a	ΔG^a	*Tech.*^{b}	*T (K)*	*Ref*
soy protein (whole)	2-Nonanone	5.5	570		1	-	(*38*)
SPI	2-Heptanone	4	110	-2781	1	298	(*37*)
	2-Octanone	4	310	-3395	1	298	(*37*)
	2-Nonanone	4	930	-4045	1	298	(*37*)
	5-Nonanone	4	541	-3725	1	298	(*37*)
	Hexanal	-	-	-825	2	363	(*6*)
	Hexanal	-	-	-1386	2	313	(*42*)
	Nonanal	4	1094	-4141	1	298	(*37*)
	Vanillin	3.18	683.5	-3696	1	285	(*23*)
β-Conglycinin (7S)	2-Nonanone	1.8	3050	-	1	-	(*38*)
	Hexanal	23	1437	-	3	293	(*39*)
	Hexanal	32	256	-3440	3	-	(*40*)
Glycinin	2-Nonanone	3.1	540	-	1	-	(*38*)

Continued on next page.

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Table II. (Continued). Binding parameters for selected flavor compounds (aldehydes and ketones) with various food proteins

Protein	*Flavor compound*	*n*[a]	*K* [a]	*ΔG*[a]	*Tech.*[b]	*T (K)*	*Ref*
(11S)	Hexanal	84	483	-	3	293	(*39*)
	Hexanal	96	270	-3690	3	-	(*40*)
Casein	Vanillin	0.66	352.66	-3322	1	285	(*23*)
WPI	2-Nonanone	1.1	370		4	-	(*33*)
	Vanillin	0.67	1713.04	-4217	1	285	(*23*)
WPC	Heptanone	0.24	4x10^7	-	3	310	(*31*)
	Octanone	0.21	4.5x10^7	-	3	310	(*31*)
	2-Nonanone	8	130	-	4	-	(*33*)
	Benzaldehyde	0.2	3.7x10^7	-	3	310	(*31*)
β-lacto-globulin	2-Heptanone	-	150	-2980	1	-	(*16*)
	2-Octanone	-	480	-3660	1	-	(*16*)
	2-Nonanone	-	2440	-4620	1	-	(*16*)
	2-Nonanone	1.1	2700	-	4	-	(*33*)
	2-Nonanone	0.2	5.3x10^7	-	3	310	(*31*)
	β-Ionone	1.08	1.7x10^6	-	5	-	(*18*)
	β-Ionone	0.8	1.9x10^6	-	5	-	(*76*)
	β-Ionone	0.85	15015	-	1	-	(*76*)
	Benzaldehyde	1	6.3x10^6	-	5	358	(*24*)
	Vanillin	1	17000	-	6	-	(*90*)
BSA	Vanillin	2	4600	-	1	298	(*89*)
	Vanillin	0.72	310000	-	6	-	(*90*)
Ovalbumin	Vanillin	0.24	4500	-	6	-	(*90*)

[a] n = number of binding sites, *K* = binding constant (M^{-1}), ΔG = free energy (cal/mol). [b] Applied technique, 1 = equilibrium dialysis, 2= IGC, 3 = headspace analysis, 4 = headspace-SPME, 5 = fluorescence spectroscopy, 6 = UV-VIS spectroscopy.

Factors Affecting Flavor-Protein Binding

In the food industry, proteins play a major role in determining the sensory and textural, as well as nutritional characteristics, of various food products. Proteins have the ability to interact with water, lipids, sugars, flavors and other ingredients. With respect to its flavor binding properties, the conformation state of a protein could have the greatest impact on its flavor binding potential (*15*). Thus, all the factors that can alter protein conformation can affect binding, including

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

temperature, pH (acid/basic), ionic strength, presence/concentration of certain chemicals and other protein modifications (*17*, *23*, *26*, *33*, *37*, *41*, *50*).

In general, heat and high pressure treatments result in changes in the secondary and tertiary structure of native proteins without breaking covalent bonds (*91*). Heat treatment is one of the most important food processing methods that can cause a change in protein functionality (*92*). Heat treatment can cause protein denaturation, which includes protein unfolding and aggregation of unfolded protein molecules (*8*). Effect of heat treatment (75 °C) on the binding of 2-nonanone to β-lagtoglobulin B was studied by O'Neill and Kinsella (*17*). They found that an increase in heating time lead to a further decrease in the binding constant. Conformation changes due to protein-protein interactions (aggregations) were indicated by changes in florescence spectra and results of non-denaturing polyacrylamide gel electrophoresis, which showed an increase in higher molecular weight proteins after heating. Chobpattana and others (*26*), who studied the effect of denaturation on the binding of vanillin to milk protein, showed that the amount of free vanillin increased significantly upon heating of BSA solutions (68 °C for 30 minutes and 75 °C for 15 minutes) as compare to non-heated BSA. The increase in free vanillin content was due to a decrease in binding affinity to vanillin caused by heat-induced structural changes in the protein.

In addition to heat treatment, low temperature can also affect flavor-protein binding. At 5 °C, tertiary and quaternary structures of soy protein can be induced to change. The binding constant of 2-nonanone to SPI at 5 °C (2000 M^{-1}) was higher than at 25 or 40 °C (930 M^{-1}) (*37*). This might be because hydrophobic interactions within the protein structure were weakened at 5 °C. The protein subunits can become reorganized within the protein molecule, thus changing the hydrophobic binding sites and resulting in higher binding affinity (*37*). Li and others (*23*) also found that decreasing temperature from 12 to 4 °C resulted in an increase in the number of binding sites in sodium caseinate, WPI and SPI, and increased the binding constants for sodium caseinate and WPI.

High pressure (HP) treatment can be used for protein modification. Recently, HP has been used for improving the functional properties of soy proteins and other food proteins (*80*, *93*). The results of these studies, which focused on flavor-protein interactions, were in general agreement in that the flavor compound structure determines its binding affinity to proteins under HP (*29*, *31*, *34*). Yang and others (*29*) modified β-lg by HP and found that the affinity towards capsaisin binding was decreased after treated with HP at 600 MPa and 50 °C for 32 min, while HP did not alter the binding of α-ionone, β-ionone, cinnamaldehyde and vanillin with β-lg. This might be because HP can cause β-lg unfolding, but may not cause an increase in surface hydrophobicity. Therefore, the binding affinity of this protein towards hydrophobic flavor compounds might not change. Later, Liu and others (*31*) studied the effect of HP on flavor-binding of WPC. Benzaldehyde and methyl ketones were the flavor compounds selected for study. They found that the number of binding sites and the binding constant of WPC changed after HP treatment (600 MPa at 50°C). They concluded that binding depended on the type and concentration of the flavor compound, and also on the holding time during HP treatment. The effect of HP (250 versus 600 MPa) on the binding of selected flavor compounds (2-nonanone, 1-nonanal and (*E*)-2-nonenal) with WPI (*34*) was studied using the three stage model developed by Considine and others (*30*). At stage I (0.1-150 MPa) the native structure of β-lg was stable; at stage II (200-450 MPa) the native monomer was interchangeable (reversible) with the non-native monomer and disulfide-bonded dimmers; and at stage III (>500 MPa) high molecular weight aggregates of β-lg were produced. The authors found that the binding of (*E*)-2-nonenal to WPI increased after treatment at 250 MPa, while the binding of 1-nonanal and 2-nonanone were not altered. For the 600 MPa treatment, the binding of (*E*)-2-nonenal continuously increased, the binding of 2-nonanone decreased, and there was no effect on the binding of nonanal. They concluded that HP affected protein-flavor interactions in accordance with flavor compound structure and suggested that hydrophobic interactions were weakened, while covalent interactions were strengthened by HP treatment.

pH can be related to flavor-protein binding because it can induce conformation changes in proteins. At neutral pH, most proteins are stable due to a small net electrostatic repulsive energy. However, the swelling and unfolding of protein molecule can occur at extremes in pH causing strong intramolecular electrostatic repulsion. Disulfide bonds in the protein molecule can be broken at alkaline pH, causing protein unfolding, which usually results in an increase in flavor binding (*94*). Zhou and others (*41*) varied the pH (4.5, 7 and 9) in the study of binding properties of 2PP to soy protein. They found that 2PP bound more strongly to soy proteins (SPI, β-conglycinin and glycinin) under basic conditions followed by neutral and then acidic conditions. Furthermore, ionic strength also affects protein conformation and thus its flavor binding ability. Guichard (*3*) found that a “salting out” effect caused a decrease in retention of benzaldehyde by β-lg. In agreement, Zhou and others (*41*) also found that binding of 2PP decreased when the concentration of NaCl was increased due to the destabilization of electrostatic interactions.

Chemical modifications such as ethylation, glycosylation and deamidation have been used to improve the functional properties of proteins. When protein side groups are modified, the generally result will be a change in the polarity and/or net charge of the protein. Therefore, protein conformation may change due to folding, unfolding and/or by aggregation with other protein molecules (*91*). O’Neill and Kinsella (*16*) studied the binding of 2-nonanone with native β-lg B versus β-lg B modified by ethylation (ethyl esterification) and reduction of disulfide bonds with sodium sulfite. Binding decreased after modification due to changes in protein conformation caused by the destabilizing effects of the esterified free carboxylic groups, thus the native form of protein unfolded and underwent hydrophobic interactions with other protein molecules (*16*). Effect of the modification of sodium caseinate by glycosylation using galactose, maltose, glucose, lactose and fructose on flavor binding was studied by Fares and others (*20*). They found that increasing of degree of modification could decrease the binding of diacetyl.

Another protein modification that affects flavor-protein binding interaction is deamidation, which is a protein hydrolysis method. It can alter secondary and tertiary structures of protein by removing amide groups. Amide groups in glutamine and asparagine residues are converted into acid residues with the

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

release of ammonia. Deamidation can be conducted both enzymatically and non-enzymatically. Enzymatic deamidation is generally more desirable than chemical modification because it is substrate specific, can be conducted under mild reaction conditions and is considered as natural and safe (*95*, *96*). However, only a few commercial enzymes are available for deamidation, including peptidoglutaminases, deamidases, transglutaminases and some proteases. Among the chemical deamidation methods, acid-catalyzed deamidation has an advantage, including milder conditions, over heat-induced deamidation, and there is no concern as is the case with base-catalyzed deamidation. Furthermore, acid deamidation is cheaper than methods that use enzymes. However, use of acid can lead to conformation changes and also to hydrolysis of peptide bonds. Lozano (*45*) studied the effect of non-enzymatic deamidation of SPI on the binding of flavor compounds using the IGC technique, they found that using sodium dodecyl sulfate (SDS) for deamidation could reduce the overall flavor binding affinity of SPI. The binding of flavor compounds to the deamidated SPI depended on the chemical characteristics of the flavor compounds tested. Binding of carbonyl containing flavor compounds to deamidated SPI were significantly decreased due to the reduction of imide formation and change in the binding mechanism to be only hydrogen bonding.

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Conclusions

Food flavor, a major determinant of consumer acceptability, is greatly affected by the presence of soy-protein. Soy proteins can impart off-flavors and/or bind desirable flavors, thus causing imbalanced flavor profiles and/or flavor fade in the food product. Significant technological advances have led to the development of soy-containing foods with improved flavors by either removal or by masking of soy-associated off-flavors. Of equal importance, and as discussed in this chapter, has been the problem of flavor binding by soy proteins. However, despite the great efforts taken to measure and understand the chemical phenomena involved in flavor-protein binding, little if any technological advances have been made towards solving this problem. More fundamental and applied research is needed to aid food manufacturers in the production of (soy) protein-enriched foods of high and acceptable flavor quality.

References

1. Plug, H.; Haring, P. *Trends Food Sci. Technol.* **1993**, *4*, 150–152.
2. Plug, H.; Haring, P. *Food Qual. Prefer.* **1994**, *5*, 95–102.
3. Guichard, E. *Food Rev. Int.* **2002**, *18*, 49–70.
4. Zhou, Q.; Cadwallader, K. R. In *Food Flavor: Chemistry, Sensory Evaluation, and Biological Activity*; ACS Symposium Series 988; Tamura, H., Ebeler, S. E., Kubota, K., Takeoka, G. R., Eds.; American Chemical Society: Washington, DC, 2008; pp 45−54.
5. Wilson, L. A. In *World Soybean Research Conference III: Proceedings*; Shibles, R., Ed.; Westview Press, Inc.: Boulder, CO, 1985; pp 158−165.

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

6. Aspelund, T. G.; Wilson, L. A. *J. Agric. Food Chem.* **1983**, *31*, 539–545.
7. O'Neill, T. E. In *Flavor-Food Interactions*; ACS Symposium Series 633; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washington, DC, 1996; pp 59−74.
8. Kühn, J.; Considine, T.; Singh, H. *J. Food Sci.* **2006**, *71*, R72–R80.
9. Gremli, H. A. *J. Am. Oil Chem. Soc.* **1974**, *51*, 95A–97A.
10. Schutte, L.; van den Ouweland, G. A. M *J. Am. Oil Chem. Soc.* **1979**, *56*, 289–290.
11. Preininger, M. In *Ingredient Interactions Effects on Food Quality*, 2nd ed.; Gaonkar, A. G., McPherson, A., Eds.; CRC Press: Boca Raton, FL, 2006; pp 477−542.
12. Damodaran, S. Amino Acids, Peptides, and Proteins. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; CRC Press: Boca Raton, FL, 1996; pp 321−429.
13. Mottram, D. S.; Szauman-Szumski, C.; Dodson, A. *J. Agric. Food Chem.* **1996**, *44*, 2349–2351.
14. Meynier, A.; Rampon, V.; Dalgalarrondo, M.; Genot, C. *Int. Dairy J.* **2004**, *14*, 681–690.
15. Damodaran, S.; Kinsella, J. E. *J. Agric. Food Chem.* **1980**, *28*, 567–571.
16. O'Neill, T. E.; Kinsella, J. E. *J. Agric. Food Chem.* **1987**, *35*, 770–774.
17. O'Neill, T. E.; Kinsella, J. E. *J. Food Sci.* **1988**, *53*, 906–909.
18. Dufour, E.; Haertlé, T. *J. Agric. Food Chem.* **1990**, *38*, 1691–1695.
19. McNeill, V. L.; Schmidt, K. A. *J. Food Sci.* **1993**, *58*, 1142–1144, 1147.
20. Fares, K.; Landy, P.; Guilard, R.; Voilley, A. *J. Dairy Sci.* **1998**, *81*, 82–91.
21. Sostmann, K.; Guichard, E. *Food Chem.* **1998**, *62*, 509–513.
22. Guichard, E.; Langourieux, S. *Food Chem.* **2000**, *71*, 301–308.
23. Li, Z.; Grün, I. U.; Fernando, L. N. *J. Food Sci.* **2000**, *65*, 997–1001.
24. Marin, I.; Relkin, P. *Food Chem.* **2000**, *71*, 401–406.
25. Fabre, M.; Aubry, V.; Guichard, E. *J. Agric. Food Chem.* **2002**, *50*, 1497–1501.
26. Chobpattana, W.; Jeon, I. J.; Smith, J. S.; Loughin, T. M. *J. Food Sci.* **2002**, *67*, 973–977.
27. Lübke, M.; Guichard, E.; Tromelin, A.; Le Quéré, J. L. *J. Agric. Food Chem.* **2002**, *50*, 7094–7099.
28. Mironov, N. A.; Breus, V. V.; Gorbatchuk, V. V.; Solomonov, B. N.; Haertlé, T. *J. Agric. Food Chem.* **2003**, *51*, 2665–2673.
29. Yang, J.; Powers, J. R.; Clark, S.; Dunken, A. K.; Swanson, B. G. *J. Food Sci.* **2003**, *68*, 444–452.
30. Considine, T.; Singh, H.; Patel, H. A.; Creamer, L. K. *J. Agric. Food Chem.* **2005**, *53*, 8010–8018.
31. Liu, X.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. *J. Food Sci.* **2005**, *70*, C581–C585.
32. Gkionakis, G. A.; Taylor, K. D. A.; Ahmad, J.; Heliopoulos, G. *Int. J. Food Sci. Technol.* **2007**, *42*, 165–174.
33. Kühn, J.; Zhu, X. -Q.; Considine, T.; Singh, H. *J. Agric. Food Chem.* **2007**, *55*, 3599–3604.
34. Kühn, J.; Considine, T.; Singh, H. *J. Agric. Food Chem.* **2008**, *56*, 10218–10224.
35. Tavel, L.; Andriot, I.; Moreau, C.; Guichard, E. *J. Agric. Food Chem.* **2008**, *56*, 10208–10217.

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

36. Damodaran, S.; Kinsella, J. E. *J. Agric. Food Chem.* **1981**, *29*, 1253–1257.
37. Damodaran, S.; Kinsella, J. E. *J. Agric. Food Chem.* **1981**, *29*, 1249–1253.
38. O'Neill, T. E.; Kinsella, J. E. *J. Food Sci.* **1987**, *52*, 98–101.
39. O'Keefe, S. F.; Resurreccion, A. P.; Wilson, L. A.; Murphy, P. A. *J. Food Sci.* **1991**, *56*, 802–806.
40. O'Keefe, S. F.; Wilson, L. A.; Resurreccion, A. P.; Murphy, P. A. *J. Agric. Food Chem.* **1991**, *39*, 1022–1028.
41. Zhou, A.; Boatright, W. L.; Johnson, L. A.; Reuber, M. *J. Food Sci.* **2002**, *67*, 142–145.
42. Zhou, Q.; Cadwallader, K. R. *J. Agric. Food Chem.* **2004**, *52*, 6271–6277.
43. Zhou, Q.; Cadwallader, K. R. *J. Agric. Food Chem.* **2006**, *54*, 1838–1843.
44. Zhou, Q.; Lee, S. -Y.; Cadwallader, K. R. *J. Agric. Food Chem.* **2006**, *54*, 5516–5520.
45. Lozano, P. R. Ph.D. Thesis, University of Illinois, Urbana, IL, 2009.
46. Ng, P. K. W.; Hoehn, E.; Bushuk, W. *J. Food Sci.* **1989**, *54*, 105–107.
47. Ng, P. K. W.; Hoehn, E.; Bushuk, W. *J. Food Sci.* **1989**, *54*, 324–325, 346.
48. Adams, R. L.; Mottram, D. S.; Parker, J. K.; Brown, H. M. *J. Agric. Food Chem.* **2001**, *49*, 4333–4336.
49. Grinberg, V. Y.; Grinberg, N. V.; Mashkevich, A. Ya.; Burova, T. V.; Tolstoguzov, V. B. *Food Hydrocol.* **2002**, *16*, 333–343.
50. Pérez-Juan, M.; Flores, M.; Toldrá, F. *J. Agric. Food Chem.* **2006**, *54*, 4802–4808.
51. Semenova, M.; Antipova, A. S.; Belyakova, L. E.; Polikarpov, Y. N.; Wasserman, L. A.; Misharina, T. A.; Terenina, M. B.; Golovnya, R. V. *Food Hydrocol.* **2002**, *16*, 573–584.
52. Semenova, M.; Antipova, A. S.; Misharina, T. A.; Golovnya, R. V. *Food Hydrocol.* **2002**, *16*, 557–564.
53. Semenova, M.; Antipova, A. S.; Wasserman, L. A.; Misharina, T. A.; Golovnya, R. V. *Food Hydrocol.* **2002**, *16*, 565–571.
54. Gianelli, M. P.; Flores, M.; Toldrá, F. *J. Agric. Food Chem.* **2005**, *53*, 1670–1677.
55. Hau, M. Y. M.; Gray, D. A.; Taylor, A. J. *Flavour Fragrance J.* **1998**, *13*, 77–84.
56. Kinsella, J. E. In *Flavor Chemistry of Lipid Foods*; Min, D., Smouse, T., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; pp 376−403.
57. Crowther, A.; Wilson, L. A.; Glatz, C. E. *J. Food Process Eng.* **1980**, *4*, 99–115.
58. Chung, S.; Villota, R. *J. Food Sci.* **1989**, *54*, 1604–1606.
59. Reineccius, G. In *Flavor Chemistry and Technology*, 2nd ed.; CRC Press: Boca Raton, FL, 2006; pp 433−465.
60. Jung, D.-M.; Ebeler, S. E. *J. Agric. Food Chem.* **2003**, *51*, 200–205.
61. Arthur, C. L.; Pawliszyn, J. *Anal. Chem.* **1990**, *62*, 2145–2148.
62. Zhang, Z.; Pawliszyn, J. *Anal. Chem.* **1993**, *65*, 1843–1852.
63. Zhang, Z.; Yang, M. J.; Pawliszyn, J. *Anal. Chem.* **1994**, *66*, 844A–853A.
64. Yang, X.; Peppard, T. *J. Agric. Food Chem.* **1994**, *42*, 1925–1930.
65. Grote, C.; Pawliszyn, J. *Anal. Chem.* **1997**, *69*, 587–596.
66. Roberts, D. D.; Pollien, P.; Milo, C. *J. Agric. Food Chem.* **2000**, *48*, 2430–2437.
67. Greene, S. A.; Pust, H. *J. Phys Chem.* **1958**, *62*, 55–58.
68. Gale, R. L.; Beebe, R. A. *J. Phys. Chem.* **1964**, *68*, 555–567.
69. Cantergiani, E.; Benczédi, D. *J. Chromatogr., A.* **2002**, *969*, 103–110.

70. Helen, H. J.; Gilbert, S. G. *J. Food Sci.* **1985**, *50*, 454–458.
71. Taylor, A. J. *Int. J. Food Sci. Technol.* **1998**, *33*, 53–62.
72. Marin, M.; Baek, I.; Taylor, A. J. *J. Agric. Food Chem.* **1999**, *47*, 4750–4755.
73. Hansen, A. P.; Heinis, J. J. *J. Dairy Sci.* **1992**, *75*, 1211–1215.
74. Hansen, A. P.; Heinis, J. J. *J. Dairy Sci.* **1991**, *74*, 2936–2940.
75. Reiners, J.; Nicklaus, S.; Guichard, E. *Lait* **2000**, *80*, 347–360.
76. Muresan, S.; van der Bent, A.; de Wolf, F. A. *J. Agric. Food Chem.* **2001**, *49*, 2609–2618.
77. Belloque, J.; Ramos, M. *Trends Food Sci. Technol.* **1999**, *10*, 313–320.
78. Jung, D. –M.; de Ropp, J. S.; Ebeler, S. E. *J. Agric. Food Chem.* **2002**, *50*, 4262–4269.
79. Jung, D.-M.; Ebeler, S. E. *J. Agric. Food Chem.* **2003**, *51*, 1988–1993.
80. Tang, C.-H.; Ma, C.-Y. *LWT−Food Sci. Technol.* **2009**, *42*, 606–611.
81. Price, N. C.; Dwek, R. A.; Ratcliffe, R. G.; Wormald, M. R. In *Principles and Problems in Physical Chemistry for Biochemist*, 3rd ed.; Oxford University Press, Inc.: New York, 2001; pp 54−73.
82. Steinhardt, J.; Reynolds, J. A. In *Multiple Equilibria in Proteins*; Academic Press, Inc.: New York, 1969; pp 10−33.
83. Scatchard, G. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660–672.
84. Klotz, I. M.; Walker, F. M.; Pivan, R. B. *J. Am. Chem. Soc.* **1946**, *68*, 1486–1490.
85. Klotz, I. M.; Urquhart, J. M. *J. Am. Chem. Soc.* **1949**, *71*, 1597–1603.
86. Guichard, E.; Etiévant, P. *Nahrung* **1998**, *42*, 376–379.
87. Yven, C.; Guichard, E.; Giboreau, A.; Roberts, D. D. *J. Agric. Food Chem.* **1998**, *46*, 1510–1514.
88. Meynier, A.; Garillon, A.; Lethuaut, L.; Genot, C. *Lait* **2003**, *83*, 223–235.
89. Burova, T. V.; Grinberg, N. V.; Grinberg, V. Y.; Tolstoguzov, V. B. *Colloids Surf., A* **2003**, *213*, 235–244.
90. Mikheeva, L. M.; Grinberg, N. V.; Grinberg, V. Ya.; Tolstoguzov, V. B. *Nahrung* **1998**, *42*, 185–186.
91. Hettiarachchy, N.; Kalapathy, U. In *Soybeans: Chemistry, Technology and Utilization*; Liu, K., Ed.; Chapman & Hall: New York, 1997; pp 379−411.
92. Boye, J. I.; Ma, C.-Y.; Harwalkar,V. R. In *Food Proteins and Their applications*; Damodaran, S, Paraf, A., Eds.; Marcel Dekker, Inc.: New York, 1997; pp 25−56.
93. Liu, X.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. *Innovative Food Sci. Emerging Technol.* **2005**, *6*, 310–317.
94. Damodaran, S. In *Food Chemistry*, 4th ed.; Damodaran, S., Parkin, K. L., Fennema, O. R., Eds.; CRC Press: Boca Raton, FL, 2008; pp 217−329.
95. Hamada, J. S. *J. Am. Oil Chem. Soc.* **1991**, *68*, 459–462.
96. Shih, F. F. In *Surface Activity of Proteins. Chemical and Physicochemical Modifications*; Magdassi, S., Ed.; Marcel Dekker, Inc.: New York, 1996; pp 91−113.

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch021

Chapter 22

Comparison of Key Aroma Components between Soymilks Prepared by Cold and Hot Grinding Methods

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Chenxing Sun, Keith R. Cadwallader, and Hun Kim*

Department of Food Science and Human Nutrition, University of Illinois,
1302 W. Pennsylvania Ave., Urbana, IL 61801
***hunkim@uiuc.edu**

Aroma components of soymilks made by two procedures, hot grind and cold grind, were isolated by solvent-assisted flavor evaporation (SAFE) and analyzed by gas chromatography-olfactometery (GCO) and GC-mass spectrometry (GC-MS). Hexanal, (*E,E*)-2,4-nonadienal, (*E,Z*)-2,4-decadienal, 1-octen-3-ol, (*E*)-2-nonenal and (*E,E*)-2,4-decadienal were identified as key aroma-active compounds in both hot grind and cold grind soymilks. With the exception of some thermally generated volatiles (e.g., 2-acetyl-1-pyrroline), the odor-activity values (OAVs) for most of the potent soymilk odorants were higher in the cold grind soymilk. The greater abundance of lipid-derived volatiles in the cold grind soymilk indicated the effectiveness of the hot grind process in reducing lipoxygenase activity during soymilk manufacture. However, the hot grind processing could not prevent the formation of all lipid-derived volatiles, therefore, other processing strategies are necessary to eliminate soymilk off-flavors.

The potential health benefits of a diet rich in soy in preventing heart disease (*1–3*), postmenopausal syndromes (*4*) and cancers, aging and osteoporosis (*5*) have led to a increase in the consumption of soy foods. Soymilk is among the most popular soy foods worldwide. Soymilk made by the traditional cooking method has been used in East Asia for centuries, but for Western markets the production process has been modified to improve acceptability. In particular, the volatile compounds are extremely important in soymilk flavor because the beany notes they impart to the product are deemed unpleasant or unacceptable to Western consumers. The volatile components of soymilk are derived enzymatically, auto-oxidatively or thermally from amino acids, proteins, carbohydrates and lipids (*6*). It has been reported that the abundance of volatile compounds in soymilk made from whole soybeans exceeds that of soymilk made from defatted soybeans or soymilk from whole soybeans in the absence of oxygen (*7*). The odorants in soy foods are derived primarily from lipoxygenases-catalyzed oxidation or auto-oxidation of linoleic and linolenic acids (*6*). Hexanal is the most commonly studied odorant since it is responsible for a beany and grassy aroma note in soymilk and has a low detection threshold (*8*, *9*). Other important soy odorants include 1-hexanol, (*E*)-2-nonenal, 1-octen-3-ol, (*E,E*)-2,4-decadienal and dimethyl trisulfide (*10*). Sulur compounds, which originated from degradation of the sulfur amino acids, such as methionine and cysteine (*6*), are not as important as the lipid-derived carbonyl compounds in overall soymilk flavor (*11*).

Raw soybeans contain only low concentrations of endogenous compounds responsible for the off-flavor; however, these off-flavor compounds form rapidly when soybeans are processed during the soaking and grinding steps of soymilk processing (*12*, *13*) and are difficult to remove due to the ability of these compounds to bind with soy proteins (*14*, *15*). The processing method can reduce or alter the odorant composition of soymilk (*11*). Soymilk production methods include heating filtered soymilk or a slurry containing insoluble residues, hot water grinding (*16*, *17*), cold water grinding, grinding with the addition of gallic acid (*18*) and blanching soaked whole soybeans by steaming or boiling before grinding (*17*). It has also been reported that eliminating oxygen during processing can substantially decrease the beany flavor of soymilk compared to aerobic processing (*19*). Although there are a number of advanced machines/processes for making soymilk (*20*), a great majority of the soymilk manufactured worldwide is done by hot water grinding or by traditional indirect cooking. Soymilk is highly nutritious and inexpensive. Soymilk might become more popular if the formation of undesirable beany off-flavor is prevented or at least minimized.

In the present study, our objectives were to characterize and compare key odorants in soymilk made by hot water grinding method versus soymilk made by cold water grinding method. Our hypothesis was that hot water grinding method could reduce the abundance of volatile compounds, especially lipid-derived compounds, in soy milk compared to the cold water grinding method.

Experimental

Materials

The soybean variety selected for this experiment was Pioneer 93B82. Fresh whole beans from the 2007 harvest were obtained from the Illinois Center for Soy Foods at the University of Illinois (Urbana, IL). A standardized soymilk procedure was adopted to consistently provide a homogeneous product for volatile analysis by eliminating possible sources of variation during its manufacture. First a pre

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

drying step at 95 °C for 10 min was applied to the beans to weaken the soybean hulls. This was necessary to minimize the damage casued by the rotating drum during dehulling. After dehulling, the beans were soaked in odorless distilled water at 4 ± 1 °C for 14 h. The soaked beans were washed with cold distilled water for 1 min, and the water was discarded. The ratio of odorless distilled water to dehulled soybeans was 1:7 w/w. For the **cold grind** treatment, the distilled water was at room temperature (~ 25 °C). A bean disintegrator BMI 300 (Beam Machines Inc., San Francisco, CA) equipped with a stainless steel mesh screen (no. 40) was applied to make the slurry. This grinding step lasted around 5 min. The obtained slurry was later filtered using a vibration screen separator KM-1-SS (Kason, Montreal, Canada) equipped with a no. 200 stainless steel mesh screen to separate the soymilk from the okara. The raw soymilk was then pasteurized by traditional batch cooking at 95 °C for 10 min. and then the heated soymilk was cooled in an ice-water bath for 20 min. The pasteurization step is necessary for inactivatation of trypsin inhibitors and insures safety and the high protein nutritive value of soymilk, while at the same time the soymilk would have a more stable shelf life (*21*). For the **hot grind** treatment, the above procedure was followed except that the distilled water added to the dehulled beans was preheated to boiling temperature.

Analytical grade reference compounds, including internal standards (2-ethylbutyric acid, 2-methyl-3-heptanone and 2,4,6-trimethylpyridine), were obtained from Aldrich Chemical Co. (St. Louis, MO). 2-Acetyl-1-pyrroline was synthesized using the procedure described by Fuganti et al. (*22*). Odorless distilled water was prepared by boiling glass-distilled water in an open flask until its volume was reduced by one-third of the original volume.

Solvent-Assisted Flavor Evaporation (SAFE)

A modified SAFE system was used to isolate the volatile compounds of both the cold grind and hot grind soymilks (*23*). This procedure was selected due to the nature of the soymilk's complex matrix. For the volatile extraction, the system consisted of two liquid nitrogen-cooled traps, a 3-L round-bottom flask and a transfer head. The system was operated under high vacuum (~ 10^{-5} Torr). A mixture of soymilk (500 mL), 50 g of NaCl and 10 µL of internal standard (IS) solution [containing 0.218 µg/µL of 2-ethylbutyric acid (acid fraction IS), 0.223 µg/µL of 2,4,6-trimethylpyridine (basic fraction IS) and 0.203 µg/µL of 2-methyl-3-heptanone (neutral fraction IS) in methanol] was introduced (dropwise) into the system at a rate of 10 mL/min. The total extraction time was approximately 120 min. After the SAFE extraction (approximately 400 mL of extract was obtained from each sample), the aqueous solution in the first trap was removed from the system and thawed overnight after addition of 50 mL of diethyl ether and 50 g of salt to promote the transfer of aroma compounds into the solvent. After recovery of the ether layer, the aqueous phase was extracted three times with 30 mL of diethyl ether. The organic phases were pooled (about 120 mL) and then concentrated at 43 °C using a Vigreux colume (150 mm x 15 mm)(Ace Glass Inc., Vineland, NJ) to a final volume of 30 mL.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Compound Class Fractionation

For acidic fraction, 20 mL of 0.1M NaOH was added to the concentrated 30 mL extract in a 250-mL separatory funnel where it was shaken for 3 min and left to rest for 3 min. The layers were separated and this process was repeated two more times in order to obtain a solvent phase (neutral and basic fraction) and aqueous phase (acidic fraction). The aqueous phase was acidified to pH 2~3 with 4M HCl and extracted (three times) with 20 mL of diethyl ether. The pooled solvent phase was collected as the acidic aroma fraction.

For neutral fraction, the solvent phase (neutral and basic fraction) was extracted (three times) with 20 mL of 0.1M HCl. The residual solvent phase comprised the neutral aroma fraction, and the aqueous phase contained the basic constituents.

For basic fraction, the aqueous basic phase (from above) was adjusted to pH 8~9 by adding 3.5 mL of 2M NaOH, and then extracted (three times) with 20 mL of ether. The solvent phase obtained from this separation was basic aroma fraction.

Acidic, neutral and basic fractions were each washed (twice) with 15 mL of saturated aqueous NaCl, and concentrated to 10 mL under a stream of purified nitrogen gas. Anhydrous sodium sulfate (2 g) was used for moisture removal. Fractions were concentrated to 250 µL prior to analysis.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Gas Chromatography-Olfactometery (GCO)

A GCO system (Agilent 6890 GC) equipped with an FID, a cool on-column injector and a sniffing port (DATU, Geneva, NY) was employed. Each fraction (2 µL) was analyzed on a polar (Stabilwax DA) capillary column (15 m × 0.32 mm i.d. ×0.5 µm film; Restek, Bellefonte, PA) and a nonpolar (DB-5MS) column (15 m ×0.32 mm i.d. ×0.5 µm film; J&W Scientific, Folsom, CA). The GC oven was programmed from 35 to 225 °C at a rate of 10 °C/min with initial and final holding times of 5 and 20 min, respectively. The carrier gas was helium at a constant flow rate of 2.2 mL/min. Detector and sniffing port temperatures were 250 °C. The end of the capillary column was split 1:1 between the flame ionization detector (FID) and sniff port. To prevent drying of the nasal mucosa, humidified air (30 mL/min) was supplied to the sniff port. Retention data were represented as retention indices (RI) based on the Van den Dool and Kratz (*24*) approach.

Gas Chromatography-Mass Spectrometry

GC-MS was performed on each fraction (1 µL) obtained from SAFE. A cold on-column (Stabilwax DA, 30 m x 0.25 mm i.d. x 0.25 µm film; Restek) method was utilized using an Agilent 6890 GC-5973N MSD system. Initial temperature of the inlet was 35°C during injection and was maintained at 3 °C above oven temperature thereafter. The oven was programmed from 35 to 225 °C at a rate of 10 °C/min with initial holding times of 5 and 25 min, respectively. Helium was used as the carrier gas at a constant rate of 1.9ml/min. The MSD was held at 280°C with an ionization voltage at 70 eV and a mass range from 33 to 350 amu.

Identification of Aroma Compounds

Positive identifications were made by comparing retention indices (RI), mass spectra and odor properties of unknowns with those of authentic standards. Tentative identifications were based on comparison of the mass spectra of unknown compounds with those in the National Institute of Standards and Technology (NIST, 1992) mass spectral database or on RI values and odor properties of unknowns matched against those of authentic standards. Retention indices were calculated by using an n-alkane series.

Quantitation of Odor-Active Compounds

Semi-quantitative analysis was conducted by assuming that the MS response factor for every odorant was 1 compared against the internal standards: 2-ethylbutyric acid, 2,4,6-trimethylpyradine and 2-methyl-3-heptanone for acidic, basic and neutral fractions, respectively. All experiments, including soymilk preparation and chemical analyses, were conducted in duplicate.

Results and Discussion

A combined of total of 32 aroma compounds were detected by GCO in the SAFE extracts from the two different soymilks (Table I). Twelve odorants [hexanal, 2-heptanone, nonanal, (*E*)-2-octenal, 1-octen-3-ol, (*E*)-2-nonenal, (*Z*)-2-nonenal, (*E,E*)-2,4-nonadienal, (*E,Z*)-2,4-decadienal, (*E,E*)-2,4-decadienal, octanoic acid and p-cresol] have been previously identified as aroma components of soymilk (*25*, *26*).

Hexanal was previously reported as the main contributor to the green/cut-grass aroma of soymilk (*27*). Based on the OAV results of the present study (Table II) it appears that hexanal is an important component of the overall soymilk aroma. However, there are several other odorants with OAVs that are close to or exceed the OAVs of hexanal. These include (*E,E*)-2,4-nonadienal, (*E,Z*)-2,4-decadienal, 1-octen-3-ol, (*E*)-2-nonenal and (*E,E*)-2,4-decadienal, which exhibited high OAVs in both hot grind and cold grind soymilks.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Table I. Aroma Compounds Determined in Hot Grind and Cold Grind Soymilks

			RI[c]		
Compound	*Odor properties*[a]	*Fr*[b]	*WAX*	*DB-5*	*ID*[d]
Ethyl acetate	solvent-like	NF	894	628	MS/RI
Pentanal	butter	NF	961	735	MS/RI/OP
Hexanal	green	NF	1075	810	MS/RI/OP
Butanol	fruity	NF	1139	675	MS/RI/OP
2-Methyl-(*E*)-2-pentenal	green, herbal	NF	1147	693	MS/OP
1-Penten-3-ol	pungent	NF	1165	683	MS/RI/OP
2-Heptanone[e]	soapy,fruity	NF	1174	891	MS/RI/OP
Heptanal[e]	fatty	NF	1176	906	MS/RI/OP
unknown	beany	NF	1185	N/A[f]	N/A[f]
(*E*)-2-Hexenal	apple-like	NF	1206	853	RI/OP
2-Pentylfuran	green-bean like	NF	1212	993	MS/RI/OP
1-Pentanol	fruity.aromatic	NF	1245	764	RI/OP
Octanal	fatty, orange	NF	1279	1003	MS/RI/OP
(*E*)-2-Heptenal	fatty,almond-like	NF	1310	956	MS/RI/OP
2,3-Octanedione	oxidized fat	NF	1319	886	MS/OP
2-Acetyl-1-pyrroline [e]	popcorn	BF	1336	915	MS/RI/OP
1-Hexanol	green,flowery	NF	1346	875	MS/RI/OP
(*Z*)-3-Hexen-1-ol	leaf-like	NF	1373	856	MS/RI/OP
Nonanal	tallowy,fruity	NF	1382	1090	MS/RI/OP
(*E*)-2-Octenal	raw peanut	NF	1414	1045	MS/RI/OP
1-Octen-3-ol	mushroom	NF	1444	1012	MS/RI/OP
(*E,E*)-2,4-Heptadienal[g]	fatty	NF	1478	1012	MS/RI/OP
(*E*)-2-Nonenal	cucumber, hay	NF	1520	1168	MS/RI/OP
1-Octanol	mushroom buttery like	NF	1548	996	MS/RI/OP
(*E,E*)-3,5-Octadien-2-one	milky,candy-like	NF	1554	1095	MS/RI/OP
(*E*)-2-Octen-1-ol	mushroom buttery like	NF	1606	983	RI/OP

Continued on next page.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Table I. (Continued). Aroma Compounds Determined in Hot Grind and Cold Grind Soymilks

Compound	*Odor properties*[a]	*Fr*[b]	*RI*[c] *WAX*	*RI*[c] *DB-5*	*ID*[d]
(*E,E*)-2,4-Nonadienal	beany, fried	NF	1684	1210	MS/RI/OP
(*E,Z*)-2,4-Decadienal	deep-fried	NF	1749	1243	MS/RI/OP
(*E,E*)-2,4-Decadienal	deep-fried	NF	1794	1340	MS/RI/OP
Guaiacol	medicine, smoky	NF	1822	1090	N/A[f]
Hexanoic acid	sweaty, ham	AF	1830	N/A[f]	MS/RI/OP
Octanoic acid	sweaty, waxy	AF	2044	N/A[f]	MS/RI/OP
p-Cresol	smoky,phenolic	NF	2051	1079	MS/RI
Nonanoic aicd	waxy, rubbery	AF	2149	N/A[f]	MS/OP

[a] Odor properties perceived during gas chromatography-olfactometery. [b] Organic fraction in which compounds was detected (NF, neutral fraction; AF, acidic fraction ;BF ,basic fraction). [c] Retention index on Stabilwax (WAX) and DB-5 columns. [d] Method of identification (MS, mass spectral match with NIST database; OP, odor property match with standard; RI, retention index match with standard). [e] Compound found in hot grind soymilk only. [f] Not available. [g] Compound found in cold grind soymilk only.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Table II. Concentrations and Odor-Activity Values of Selected Odorants in Hot Grind and Cold Grind Soymilks

		Cncn (ug/L) [a]			*OAV*[c]	
Compound	*Odor properties*	*Hot*	*Cold*	*Threshold (µg/L)*[b]	*Hot*	*Cold*
Pentanal	butter	11.9 ± 2.2	21.7 ± 1.0	12	~1	2
Hexanal	green	242 ± 61	264 ± 32	4.5	54	59
2-Methyl-(*E*)-2-pentenal	green,herbal	24.0 ± 6.7	27.2 ± 2.5	12	2	2
2-Heptanone[d]	soapy,fruity	9.9 ± 1.5	nd [e]	5	2	--
Heptanal[d]	fatty	8.4 ± 0.4	nd [e]	3	3	--
2-Pentylfuran	green-bean like	14.3 ± 0.6	18.8 ± 0.7	6	2	3
1-Pentanol	fruity.aromatic	57.8 ± 3.1	84.6 ± 8.1	12	5	7
Octanal	fatty	4.1 ± 1.1	14.4 ± 1.6	0.7	6	21
(*E*)-2-Heptenal	fatty,almond-like	13.4 ± 0.8	43.1 ± 3.7	13	1	3
2,3-Octanedione	oxidized fat	15.4 ± 0.8	41.0 ± 8.4	12	1	3
2-Acetyl-1-pyrroline[d]	popcorn	2.1 ± 0.5	nd[e]	0.1	21	--
1-Hexanol	green,flowery	65.4 ± 8.2	66.5 ± 5.2	50	1	1
Nonanal	tallowy,fruity	26.3 ± 1.0	65.9 ± 8.6	1	26	67
(*E*)-2-Octenal	raw peanuts	11.1 ± 1.8	30.2 ± 3.8	4	3	8
1-Octen-3-ol	mushroom	117 ± 19	221 ± 16	1	115	223
(*E,E*)-2,4-Heptadienal[f]	fatty	N/A[e]	40.6 ± 2.0	3	--	14
(*E*)-2-Nonenal	cucumber,hay	4.8 ± 1.1	20.4 ± 1.7	0.15	30	136

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

		Cncn (ug/L) [a]		*Threshold (µg/L)*[b]	*OAV*[c]	
Compound	*Odor properties*	*Hot*	*Cold*		*Hot*	*Cold*
(*E,E*)-2,4-Nonadienal	beany	13.2 ± 0.5	68.9 ± 2.1	0.09	143	768
(*E,Z*)-2,4-Decadienal	deep-fried	12.4 ± 0.7	47.2 ± 5.0	0.07	177	671
(*E,E*)-2,4-Decadienal	deep-fried	102 ± 5.6	225 ± 30	0.07	1441	3191
Hexanoic acid	sweaty, ham	1520 ± 24	1296 ± 56	290	5	4

[a] Average (n=2) concentration (microgram per liter ± standard deviation); Hot: hot grind soymilk; Cold: cold grind soymilk. [b] Odor detection threshold in water (from reference (*28*)). [c] Odor-activity value (concentration/threshold). [d] Compound detected in hot grind soymilk only. [e] Not detected. [f] Compound detected in cold grind soymilk only.

The above mentioned compounds can be derived via the enzymatic degradation of lipids. Lipoxygenase, hydroperoxidelyase, *Z/E*-enal isomerase and alcohol dehydrogenase enzymes play important roles in the formation of volatile compounds in plants (*29*). Lipxoygenase catalyzes the formation of hydroperoxides of unsaturated fatty acids (e.g., linoleic and linolenic acids). The hydroperoxides are then converted to secondary oxidation products including aldehydes, alcohols, or acids by the hydroperoxide lyases and other enzymes (*30*, *31*). (*E*)-2-Nonenal was reported previously as a product of the thermal decomposition of hydroperoxides from linoleic and linolenic acids (*32*). Among the most significant oxidation products formed from linoleic acid, hexanal and 1-hexanol impart green, grassy and beany odors (*30*, *33*).

2–Pentylfuran might contribute to typical beany flavor in the soymilk due its characteristic bean-like aroma note. 2–Pentylfuran was previously reported as a volatile component of soybean oil containing 5 ppm chlorophyll b and stored under light for 96 h (*34*). However, the concentration and OAV of 2–pentylfuran in soymilk was relatively low in the soymilks analyzed in the present study (Table II). The chemical mechanism for the formation of 2–pentylfuran from linoleic acid by singlet oxygen was discussed by Bradly and Min (*35*). Singlet oxygen could be formed in the presence of singlet oxygen sensitizers such as chlorophyll or riboflavin in soymilk and atmospheric oxygen under light (*35*). (*E*)-2–Heptenal was also formed by singlet oxygen from linoleic acid (*36*).

In the hot grind soymilk the formation of heterocyclic compounds such as 2-acetyl-1-pyrroline can cause an increase in the overall roasted aroma intensity. Although, in the present study, this compound was below MS detection limits, it would be expected to contribute more to the overall aroma of the hot grind soymilk since it received a greater degree of thermal treatment than the cold grind soymilk. 2-Acetyl-1-pyrroline was previously reported as a main contributor to the "burnt flavor" of roasted soybeans (*37*) and "cooked flavor" in UHT-processed cow's milk. (*38*). However, 2-acetylthiazole and 2-acetyl-2-thiazoline, which were reported in soymilk treated under UHT conditions (*26*), were not detected (by GCO or GC-MS) in either of the soymilks in the present study.

The OAV is the ratio of the concentration of an odorant to its threshold, and can reflect the flavor significance from a sensory perspective (*39*). There were twenty-one aroma compounds with OAVs greater than unity in both soymilk samples (Table II), indicating they may make some contribution to the overall aromas of the soymilks. In particular, (*E,E*)-2,4-decadienal, (*E,Z*)-2,4-decadienal, (*E,E*)-2,4-nonadienal, 1-octen-3-ol, hexanal and (*E*)-2-nonenal had much higher OAVs than the other odorants detected in both the hot grind and cold grind soymilks.

(*E,E*)-2,4-Decadienal has a fried, fatty aroma note. The odor detection threshold value for this compound is very low (0.07 ppb in water) (*40*). This might explain why (*E,E*)-2,4-decadienal had the highest OAV in our study.

In our study, 2-acetyl-1-pyrroline was only found in hot grind soymilk sample and its concentration was low, but it still contributed greatly (due to its very low odor detection threshold of 0.1 ppb (*40*)), to the roasted and popcorn aroma of hot grind soymilk.

By comparing the OAVs between hot grind and cold grind soymilks, we found that the OAVs of most aroma compounds in the hot grind soymilk were lower

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

than that in cold grind soymilk sample, especially (*E,E*)-2,4-decadienal, (*E,Z*)-2,4-decadienal, (*E,E*)-2,4-nonadienal, 1-octen-3-ol and (*E*)-2-nonenal, which had much lower OAVs in hot grind soymilk than in cold grind soymilk.

It is well-known that (*E,E*)-2,4-decadienal, (*E,Z*)-2,4-decadienal and (*E,E*)-2,4-nonadienal are products of the oxidation of unsaturated fatty acids catalyzed by lipoxygenase. Lipoxygenase can be deactivated when the temperature is higher than 80 °C (*41*). Therefore, during the hot grinding process (boiling water, ~100 °C), most of the lipoxygenase activity is lost. Lipoxygenase-deficient soybean varieties have been shown to produce soymilk with less off-flavor than normal soybean varieties (*11*). Kobayashi et al. (*16*) compared the aroma compositions of soymilk made from the Suzuyutaka (normal lipoxygenases variety), Yumeyutaka (lacking lipoxygenases 2 and 3) and Kyushu No. 111 (lacking lipoxygenases 1, 2 and 3). They found soymilk without lipoxygenases 2 and 3 or without lipoxygenases 1, 2 and 3 produced less overall volatile components than the normal-lipoxygenase Suzuyutaka soybean. Hexanal and hexanol were found in relatively low abundance in the lipxoxygenase-deficient varieties. In addition, the concentrations of 1-octen-3-ol, (*E*)-2-nonenal and (*E,E*)-2,4-decadienal were still present in relatively high abundance in soymilks made from the two lipoxygenase-lacking varieties. Feng et al. (*19*) also found that controlling the atmospheric oxygen provided a means to control the formation of beany flavor compounds, e.g. they observed a decline in hexanal. However, threre were still some lipid oxidation products found in even anaerobically produced soymilk because of lipid autoxidation that can occur during the post harvest handling of soybeans (*19*). In our study, 1-octen-3-ol, (*E*)-2-nonenal and (*E,E*)-2,4-decadienal were detected in the hot grind soymilk, which means these compounds could also be formed by an oxidation pathway other than lipoxygense oxidation pathway or perhaps by thermally resistant lipoxygenase.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Conclusions

Hexanal, (*E,E*)-2,4-nonadienal, (*E,Z*)-2,4-decadienal, 1-octen-3-ol, (*E*)-2-nonenal and (*E,E*)-2,4-decadienal were important aroma compounds in both hot grind and cold grind soymilk. Hot grind processing could reduce flavor intensity of soymilk, because lipoxygenase could be deactivated during hot grinding. However, hot grind processing could not eliminate all of the soy-associated flavors. Therefore, other processing stratages (such as elimination of oxygen) should be combined with the process.

Acknowledgments

We would like to acknowledge the support of the China Agricultural University-University of Illinois at Urbana-Champaign Corporate Fellowship Program for financial support, and the Illinois Center for Soy Foods at the University of Illinois for help with the soy milk processing experiments.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

References

1. Food Labeling: Health Claim; Soy Protein and Coronary Heart Disease. FDA, 21 CFR Pt. 101. Final Rule. *Fed. Regist.* **1999**, *64* (206), 57700−57733.
2. Anderson, J. W.; Smith, B. M.; Washnock, C. S. *Am. J. Clin. Nutr.* **1999**, *70*, 464S–474S.
3. Liu, K. *Food Technol.* **2000**, *54* (7), 46–58.
4. Friedman, M.; Brandon, D. L. *J. Agric. Food Chem.* **2001**, *49*, 1069–1086.
5. Ikeda, Y.; Iki, M.; Morita, A.; Kajita, E.; Kagamimori, S.; Kagawa, Y.; Yoneshima, H. *J. Nutr.* **2006**, *136*, 1323–1328.
6. MacLeod, G.; Ames, J. *Crit. Rev. Food Sci. Nutr.* **1988**, *27* (4), 219–400.
7. Min, S.; Yu, Y.; Yoo, S.; Martin, S. St. *J. Food Sci.* **2005**, *70*, C1–C7.
8. Wilkens, W. F.; Lin, F. M. *J. Agric. Food Chem.* **1970**, *18*, 333–336.
9. Guadagni, D. G.; Buttery, R. G.; Turnbaugh, J. G. *J. Sci. Food Agric.* **1972**, *23*, 1435–1444.
10. Ho, C. T.; Zhang, Y.; Shi, H.; Tang, J. *Food Rev. Int.* **1989**, *5*, 253–287.
11. Yuan, S. H.; Chang, K. C. *J. Agric. Food Chem.* **2007**, *55*, 426–431.
12. Blasé, R. J. Factors Influencing Endogenous Flavor Compounds in Soybeans. Ph.D. Thesis, Clemson University, Clemson, SC, 1990.
13. Ha, E. Y. W.; Morr, C. V.; Seo, A. *J. Food Sci.* **1992**, *57*, 414–417, 436.
14. Aspelund, T. G.; Wilson, L. A. *J. Agric. Food Chem.* **1983**, *31*, 539–545.
15. O'Keefe, S. F.; Resurreccion, A. P.; Wilson, L. A.; Murphy, P. A. *J. Food Sci.* **1991**, *56*, 802–806.
16. Kobayashi, A.; Tsuda, Y.; Hirata, N.; Kubota, K.; Kitamura, K. *J. Agric. Food Chem.* **1995**, *43*, 2449–2452.
17. Endo, H.; Ohno, M.; Tanji, K.; Shimada, S.; Kaneko, K. *Food Sci. Technol. Res.* **2004**, *10*, 328–333.
18. Boatright, W. L. *J. Am. Oil Chem. Soc.* **2002**, *79*, 317–323.
19. Feng, Y. W.; Acree, T. E.; Lavin, E. H. In *Aroma Active Compounds in Foods*; ACS Symposium Series 794; Takeoka, G. R., Guntert, M., Engel, K.-H., Eds.; American Chemical Society: Washington, DC, 2001; pp 251−264.
20. Chen, M. Y. B.; Wei, L. S.; Nelson, A. I. *J. Food Sci.* **1989**, *54*, 963–967.
21. Yuan, S. H.; Chang, K. C. *J. Agric. Food Chem.* **2008**, *56*, 7957–7963.
22. Fuganti, C.; Gatti, F. G.; Serra, S. *Tetrahedron* **2007**, *63*, 4762–4767.
23. Engel, W. B.; Schieberle, P. *Eur. Food Res. Technol.* **1999**, *209*, 237–241.
24. Van den Dool, H.; Kratz, P. D. *J. Chromatogr.* **1963**, *11*, 463–471.
25. Benitez, D. Effect of Presoak Treatment (Blanching vs. Cold Soaking) and Sodium Bicarbonate Addition during Processing on Volatile Composition of Soymilk. MS. Thesis, University of Illinois, Urbana, IL, 2003, pp 69−80.
26. Lozano, P.; Cadwallader, K. R. *J. Agric. Food Chem.* **2007**, *55*, 3018–3026.
27. Torres-Penaranda, A. V.; Reitmeier, C. A. *J. Food Sci.* **2001**, *66*, 352–356.
28. Rychlik, M.; Schieberle, P.; Grosch, W. *Compilation of Odor Threshold, Odor Qualities and Retention Indices of Key Food Odorants*; Deutsche Forschungsanstalt für Lebensmittelchemie and Institut für Lebensmittelchemie der Technischen Universität München: Garching, Germany, 1998; pp 7−45.

29. Gardner, H. W. In *Flavor Chemistry of Fats and Oils*; Min, B. D., Smouse, T. H., Eds.; American Oil Chemists' Society: Champaign, IL, 1985; pp 189–206.
30. Hildebrand, D. F. *Physiol. Plant.* **1989**, *76*, 249–253.
31. Gardner, H. W. In *Flavor Chemistry of Lipid Foods*; Min, D. B., Smouse, T. H., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; pp 98−112.
32. Frankel, E. N.; Neff, W. E. *Lipids* **1981**, *16*, 279–283.
33. Wang, Z. H.; Macura, D. D.; Durance, T. D.; Nakai, S. *Food Res. Int.* **1998**, *30*, 503–511.
34. Min, D. B.; Callison, A. L.; Lee, H. O. *J. Food Sci.* **2003**, *68*, 1175–1178.
35. Bradley, D. G.; Lee, H. O.; Min, D. B. *J. Food Sci.* **2003**, *68*, 491–494.
36. Lee, J. Y.; Min, S.; Choe, E. O.; Min, D. B. *J. Food Sci.* **2003**, *68*, 1933–1937.
37. Kato, H.; Doi, Y.; Tsugita, T.; Kosai, K.; Kamiya, T.; Kurata, T. *Food Chem.* **1981**, *7*, 87–94.
38. Colahan-Sederstrom, P.; Peterson, G. D. *J. Agric. Food Chem.* **2005**, *53*, 398–402.
39. Acree, T. E.; Barnard, J.; Cunningham, D. G. *Food Chem.* **1984**, *14*, 273–286.
40. Buttery, R. G.; Orts, W. J.; Takeoka, G. R.; Nam, Y. *J. Agric. Food Chem.* **1999**, *47*, 4353–4356.
41. Wilkens, W. F.; Mattick, L. R.; Hand, D. B. *Food Technol.* **1967**, *21*, 1630–1633.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch022

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Chapter 23

Volatile Flavor Compounds and Flavor Profiles of Thai Soy Sauce

Sittiwat Lertsiri,*,1 Pitipong Wanakhachornkrai,1 Apinya Assavanig,1 Siree Chaiseri,2 and Thongchai Suwonsichon3

[1]Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand
[2]Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand
[3]Department of Product Development, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand
***scsla@mahidol.ac.th**

Soy sauce is fermented soybean condiment originally invented in China from the ancient time. Since this product has been spread widely in Asia, each region has developed its own recipe and fermenting process, leading to varieties of product flavor profiles. In this work, the volatile flavor compounds and their relationship with flavor profiles of Thai soy sauce were studied. As a result, 60 odor-active compounds have been characterized by gas chromatography-mass spectrometry (GC-MS) and GC-olfactometry. The odor activities of those compounds were further investigated by approaches of Aroma extract dilution analysis, Dynamic headspace dilution analysis, and Calculation of odor activity value. We concluded that 2-methyl propanal, 1-propanol, 3-methyl-1-butanol, 2,6-dimethyl pyrazine, mathional, 3-methyl butanoic acid, 2-methoxyphenol, 3-hydroxy-2-methyl-4H-pyran-4-one, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone were key aroma compounds of Thai soy sauce. Apart from chemical analytic work, modified quantitative descriptive analysis of 18 Thai soy sauces was conducted to understand their flavor profiles.

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Soy sauce is a fermented soybean condiment consumed worldwide. This kind of products has been originated in China as first recorded 2700 years ago. The production of soy sauce has been transferred to Asian countries, especially; Japan has long history for soy sauce production and development, for more than 1300 years (*1*). Soy sauce is made from main raw materials of soybeans, wheat, and brine; and classified into Chinese-type and Japanese-type based on the amount of wheat used (*2*). In Japanese-type soy sauce, soybeans and wheat are used at equal amount while less wheat or cereal is used in Chinese-type. Thai soy sauce is produced as the Chinese type, in small to medium-scale manufacturers, with production know-how passed from generation to generation. The mixture of cooked soybean and wheat or rice flour (7:1 ratio) is inoculated with koji starter (*Aspergillus oryzae*). After fermentation (40-48 h), the resulting koji is then immersed in a brine solution with the ratio of koji to brine of 1:3 (w/v). This gives moromi with 18-22% salt concentration. After the moromi fermentation is completed by lactic acid bacteria (*Tetragenococcus halophilus*) and halotolerant yeast (*Zygosaccharomyces rouxii* and *Candida* species), soy sauce is separated, filtered, and pasteurized before bottling (*3*, *4*).

The characteristic flavor-aroma formation in the soy sauce depends on the manner of production, raw materials and strains of microorganism used (*2*). Since this kind of products has been widely consumed in Asia, each region has developed its own recipe and fermenting process, hence varieties of soy sauce flavor characteristics from various origins are evident. Up to now, investigations on volatile constituents of soy sauce in various countries, *i.e.* Japan (*5*, *6*), Korea (*7*), Indonesia (*8*), and China (*9*) have been reported. Previously, we have indentified 93 volatile compounds in Thai soy sauce by GC-MS with various sample preparation techniques, *i.e.* dynamic headspace sampling, direct solvent extraction, and vacuum simultaneous steam distillation-solvent extraction, in order to cover wide range of their volatility (*10*). In this work, we further determined odor activities of volatile compounds being present in Thai soy sauce based on their concentration and gas chromatography-olfactometry methodology. The results from instrumental analysis and sensory descriptive analysis revealed impact of the odor-active compounds and their contribution to flavor profile. The knowledge obtained would be applicable for manufacturing optimization and quality standardization of Thai soy sauce fermentation.

Materials and Methods

Materials

Thai soy sauces were purchased from local markets in Thailand. Samples were stored in the dark at room temperature.

Organic solvents were purchased from Merck (Darmstadt, Germany) and redistilled prior to use. Internal standard and authentic reference compounds were obtained from Aldrich Chemical (St. Louis, MO). Other chemicals were of the best grade available supplied from Merck (Darmstadt, Germany).

Aroma Extract

In order to extract wide range of volatile compounds, soy sauce sample (25 g) was saturated with sodium chloride and adjusted to pH 3.0 with hydrochloric acid. Then, the sample was extracted with dichloromethane (3 times x 20 mL). After solvent layer was recovered, the sample was re-adjusted to pH 12.0 with sodium hydroxide solution and was extracted as described above. The solvent layer pool was subjected to vacuum distillation with liquid nitrogen cold trap for 4 h (*10*). Distillate obtained was frozen overnight to remove water. After concentrating with gentle nitrogen stream to 5 mL, the distillate was dried over anhydrous sodium sulfate and further concentrated to 50 μL prior to gas chromatography-mass spectrometry (GC-MS) and aroma extract dilution analysis (AEDA).

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Dynamic Headspace (DHS) Analysis

Soy sauce sample with internal standard of 2-methyl-3-heptanone at the final concentration of 152.6 ng/g was saturated with sodium chloride prior to DHS and dynamic headspace dilution analysis (DHDA). A sampling tube (15.2 x 1.6 cm i.d.) contained 5 ml of the sample was connected to a Tekmar Dohrmann 3100 purge and trap concentrator (Tekmar, Cincinnati, OH). The sample was preheated for 2 min at 40 °C, and was purged with ultra high-purity helium (50 mL/min) for 20 min to a Tenax TA trap. The trap was then dry-purged for 7 min prior to desorption of volatiles at 220 °C for 2 min. The volatiles desorbed were directly transferred onto gas chromatography-mass spectrometry (GC-MS) via electric pressure control-volatiles interface with split ratio of 10:1 (*10*).

Gas Chromatography-Olfactometry (GC-O)

The system, Agilent 6890 GC (Agilent, USA) with HP-FFAP column (25 m x 0.32 mm i.d. x 0.50 μm film thickness) and a flame ionization detector (FID), was equipped with a sniffing port (ODO II, SGE Incorporation, USA). Effluent from a GC column was split at 1:5 ratio to the FID and sniffing port. Oven temperature was hold at 40 °C for 4 min and rose at 10 °C/min to 200 °C and maintained for 20 min. The carrier gas was ultra high-purity helium at a constant flow of 1.5 mL/min. Sniffing was performed by two panelists. Aromagram indicated odor description, retention time, and odor intensity (*i.e.* very strong, strong, medium, weak and very weak). Two GC-O experiments, *i.e.* AEDA (*11*, *12*) and DHDA (*13*, *14*) were performed. For AEDA, the aroma extract were stepwise diluted 1:5 in dichloromethane to obtain dilutions of 5, 25, and 125 folds of the initial aroma extract. Flavor dilution factor (FD value) was defined as the highest dilution at which odorants was detected and represented as $\log_5$ FD. DHDA was conducted by combining dynamic headspace acquired GC-O with stepwise reducing purge volume of DHS, *i.e.* 800 mL (default value), 200 mL, 50 mL, and 12.5 mL as dilution steps. Odor potency of the odorant was determined as minimal purge volume of DHS.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed using the Agilent 6890 Plus GC/HP 5973 MSD, equipped with an HP-FFAP column as described above. One microlitre of aroma extract was injected at 230 °C with splitless mode. The GC oven temperature was programmed from 45 °C to 220 °C for HP-FFAP at the rate of 15 °C/min. The initial and final hold times were 2 and 11.40 min, respectively. For DHS-GC-MS, the GC oven temperature was held at 45 °C for 5 min, then programmed from 45 to 180 °C at 10 °C /min, following with 180 °C to 240 °C at 20 °C/min. DHS analysis of each sample was performed in duplicate. The carrier gas was ultra high- purity helium at a constant flow of 1.5 mL/min. Capillary direct-interface temperature was 280 °C. Ionization energy was 70 eV. Mass range was 35 to 450 a.m.u. (3.50 scan/s) for direct injection of the aroma extract or 20 to 350 a.m.u. (4.33 scan/s) for DHS analysis (*10*).

Compounds Identification

Positive identification of a component was performed by comparison of its retention index (RI), mass spectrum, and odor quality with an authentic reference compound or data available. Tentatively identified compounds were uniquely identified on the basis of the mass spectra from the Wiley 275.L mass spectral database (Hewlett-Packard Co.).

Quantification of Volatile Compounds

The standard addition method was used for quantification. Calibration curves were constructed by spiking 50 μL of reference solutions to make final concentration of 625-5000 ng/g and 10 μL of 2-methyl-3-heptanone solution as the internal standard to make final concentration of 152.6 ng/g in 50 g of soy sauce samples. After saturating with sodium chloride, samples were subjected to DHS analysis as mentioned above. Compounds were quantified on the basis of selected ions from MS data recorded in a scan mode (*15*). The standard curves were obtained with good linearity ($r^2 > 0.99$). The concentration of each volatile compound was then applied to calculate odor activity values (*11*).

Sensory Descriptive Analysis for Flavor Profile of Thai Soy Sauces

Flavor profiles of 18 samples of Thai soy sauce were evaluated using modified quantitative descriptive analysis method (*16*, *17*). The analysis embodied a screening criterion, development and defining sensory attributes, training, monitoring and testing the panellists' accuracy, and quantitative scoring of sample characteristics. Panellists were recruited among members of the Department of Product Development, Faculty of Agro-Industry, at Kasetsart University and trained for 3 months. Soy sauce attributes were developed based on agreement among the panellists. Fourteen sensory attributes derived from consensus were 6 aroma attributes, 4 flavor attributes and 4 taste attributes. The definitions of these attributes were described in Table I. A scoring card was developed with a 15-cm line scale marked with directional terms or anchor words (none to extreme) from each end. The samples were also determined their volatile flavor compounds by DHS-GC-MS as mentioned above.

Table I. Definition of Attributes of Sensory Descriptive Analysis

Attributes	*Descriptives*
Aroma	
Sweet	Sweet aroma of palm sugar
Molasses-like	Molasses odor
Alcohol	Pungent odor of alcohol
Soy bean paste-like	Odor of soy bean paste
Sour	Pungent odor of vinegar
Fishy	Fish sauce-like odor
Flavor	
Sweet	Sweet flavor of cooked soy bean
Sour sauce-like	Red vinegar flavor
Soy bean paste-like	Soy bean paste flavor
Fishy	Fish sauce-like flavor
Taste	
Sweet	Sweetness of sugar
Salty	Salty taste
Sour	Sour taste of vinegar
Bitterness	Bitter taste
After taste	
Salty	Left over of salty taste

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Table II. Odor-active compounds and their odor activities in aroma extract of three soy sauce samples (A, B, and C)

Calculated RI[a]	*Compound*	*Odor quality*[b]	*$\log_5$ FD factor*		
			A	**B**	**C**
> 1000	2-Methyl propanal	green, flowery	0	1	1
> 1000	2 and 3-Methyl butanal	malty	0	1	0
1001	Ethanol	ethanol-like	1	1	nd
1013	4-Methyl-2-pentanone	sweet	0	2	1
1201	Unknown	sulfury	nd	1	nd
1222	2 and 3-Methyl butanol	malty	0	1	0
1304	3-Hydroxy-2-butanone	buttery	nd	1	nd
1316	4-Methyl-2-pentanol*	sweet	nd	1	nd
1349	2,6-Dimethyl pyrazine	cooked rice-like, nutty	2	3	2
1414	Unknown	moldy	1	1	2
1437	3-Ethyl-2,5-dimethylpyrazine	cooked rice-like, nutty	nd	1	nd
1451	2-Ethyl-3,5-dimethylpyrazine	roasted	nd	nd	0
1465	Acetic acid	sour	2	2	1
1476	Methional	brothy, cooked potato	3	3	3
1484	2,3-Diethyl-5-methylpyrazine	boiled potato	1	2	1
1542	Unknown	sweet	1	0	nd
1548	Propionic acid	sour	nd	nd	0
1580	2-Methyl propanoic acid	sour, cheesy	1	2	1
1620	Dihyro-3-methyl-2(3H)-furanone	caramel-like	1	2	nd
1640	Butanoic acid	sweaty	1	2	1
1646	Phenylacetaldehyde	honey-like	nd	nd	1
1662	Benzeneacetaldehyde	dry rose-like	2	2	1
1680	3-Methyl butanoic acid	sweaty	3	3	3
1737	Methionol	meaty	3	2	1
1741	Unknown	rubber-like	0	nd	2
1745	Unknown	plastic-like	nd	nd	1
1750	Unknown	rubber-like	nd	1	nd
1751	Unknown	cresol-like	nd	1	nd
1790	Unknown	catty	nd	2	nd
1805	3-Methyl pentanoic acid*	sour	nd	nd	1
1821	Unknown	plastic-like	nd	nd	1
1834	Unknown	fermented	3	nd	2
1845	2-Hydroxy-3-methyl-2-cyclopenten-1-one*	caramel-like	3	1	1
1859	3,4-Dimethyl cyclopentenolone	caramel-like	3	3	3
1880	2-Methoxyphenol	clove-like	3	3	3
1918	Unknown	caramel-like	nd	nd	2
1929	Benzeneethanol	phenolic, spicy	3	2	3
1984	3-Hydroxy-2-methyl-4H-pyran-4-one	caramel-like	3	2	2
2025	2-Ehyl-3-hydroxy-4-pyranone	caramel-like	nd	nd	2
2026	Phenol	phenol	1	2	nd
2047	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	caramel-like	3	3	3
2064	4-Ethyl-2-methoxy-phenol	phenolic, smoke	1	2	2
2033	4-Hydroxy-2-ethyl-5-methyl-3(2H)-furanone	caramel-like	0	1	3
2105	Unknown	flowery, sweet	0	0	1
2126	2,6-Di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadene-1-one*	woody	3	2	1
2205	4-Ethyl phenol	phenolic	1	2	2
2215	4-Vinyl-2-methoxyphenol	clove-like	3	3	3
2244	Unknown	soapy	3	2	2
2270	Unknown	rubber-like	nd	1	1
2295	2,6-Dimethoxy phenol*	clove-like	1	nd	1
2359	Unknown	pleasant	nd	nd	1
2414	Unknown	pleasant	nd	1	nd
2555	2-Phenylacetic acid	chocolate-like	0	2	1
2579	Benzoic acid	dry rose-like	nd	2	2
2587	Vanillin	vanilla-like	3	3	1

[a]Retention index calculated from GC-O. [b]Quality attribute perceived on GC-O. *Tentatively identified compound. nd, not detected.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Table III. Odor-active compounds and their odor activities based on DHDA and OAV in two soy sauce samples (A and B)

Calculated RI[a]	*Chemical compound*	*Odor descriptive*[b]	Soy sauce A			Soy sauce B		
			Minimal purged volume (ml)[c]	*concentration (ng/g)*	*Calculated OAV*[d]	*Minimal purged volume (ml)*[c]	*concentration (ng/g)*	*Calculated OAV*[d]
> 900	Dimethyl sulfide	sulfury	800	1845.61	1845.6 - 6152.0	nd	nd	
> 900	2-Methyl propanal	flowery	12.5	471.59	47.2 - 486.2	12.5	524.15	52.4 - 540.4
914	2-Methyl butanal	malty	12.5	699.72	189.1 - 699.7	12.5	997.64	269.6 - 997.6
926	3-Methyl butanal	malty	12.5	222.54	71.8 - 1112.7	12.5	484.8	156.4 - 2424
940	Unknown	brothy	nd	nd		800	dn	
954	Unknown	brothy	200	dn		800	dn	
967	Ethyl propionate	flowery	12.5	27.85	1.4 - 2.8	12.5	16.83	0.8 - 1.7
995	2,3-Butanedione	buttery	nd	45.71	3.0 - 11.3	50	55.5	3.7 - 13.9
1045	1-Propanol	sweet, flowery	12.5	6776.61	0.8 - 1.0	12.5	1772.17	0.2 - 0.3
1154	1-Butanol	nd	nd	2131.65	1.7 - 4.3	nd	267.96	0.2 - 0.5
1067	2,3-Pentanedione	sweet	200	12.5	0.4	12.5	20.22	0.7
1082	Dimethyl disulfide	nd	nd	6.27	0.5 - 39.2	nd	21.93	1.8 - 137.1
1105	2-Methyl-1-propanol	nd	nd	11668.22	11.7	nd	2353.45	2.4
1208	3-Methyl-1-butanol	malty	12.5	6697.79	3.7 - 26.8	12.5	3478.49	1.9 - 13.9
1291	3-Hydroxy-2-butanone*	ether-like, solvent	nd	4129.04	0.8 - 5.2	12.5	2981.76	0.6 - 3.7
1340	2,6-Dimethyl pyrazine	cooked rice-like, nutty	200	91.42	0.002 - 0.5	200	329.95	0.5
1465	Acetic acid	sour	200	dn		800	dn	
1476	Methional	brothy	12.5	84.4	46.9 - 422.0	12.5	297.52	165.3 - 1487.6
1580	2-Methyl propionic acid	nd	nd	1485.33	0.2 -29.7	nd	3879.02	0.5 - 77.6
1640	Butanoic acid	nd	nd	nd		nd	4239.57	2.0 - 84.8
1663	Benzeneacetaldehyde	dry rose-like	nd	nd		200	dn	
1680	3-Methyl butanoic acid	cheesy	200	919.52	0.6 - 7.0	50	2057.87	1.3 - 15.6
1687	2-Furanmethanol	nd	nd	5107.14	2.6 - 5.1	nd	8045.49	4.0 - 8.0
1737	Methionol	nd	nd	nd		nd	1910.65	382.5
1898	2-Methoxyphenol	clove-like	200	85.32	28.4 - 34.1	12.5	305.9	102.0 - 122.4
2026	Phenol	nd	nd	20.55	0.003	nd	58.75	0.01
2205	4-Ethyl phenol	nd	nd	nd		nd	155.13	0.004

[a] Retention index calculated from GC-O. [b] Quality attribute perceived on GC-O. [c] Minimal purged volume on DHS. [d] OAV calculated based on odor threshold value available in Reference *20*.
*Tentatively identified compound. nd, not detected. dn, data not available.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Statistical Analysis

Cluster analysis was applied to categorize soy sauce samples based on sensory evaluation. Principal component analysis (PCA) was applied to determine correlation of sensory attributes and peak area of volatile compounds obtained from DHS-GC-MS, based on Pearson correlation matrix with a level of significance at 95%.

Results and Discussion

GC-O Experiments and Odor Activity Values

We totally found 60 odor-active compounds on aromagrams of aroma extract and DHS obtained from all samples analyzed. From first screening, 29 odor-active compounds were detected in all aroma extract of three samples (Table II). Among these, methional and 2-methyl propanal were not identified in our previous work (*10*). This was due to the peaks area of these compounds was below target minimal peak area for detection above 10^5 and 10^4 counts for direct solvent extraction and DHS, respectively. However, in this work, GC-O revealed occurrence of methional and 2-methyl propanal as important and common odor-active compounds found in all samples tested. By AEDA, the most potent odorants which gave the highest $\log_5$ FD factor were methional, 3-methyl butanoic acid, 3,4-dimethyl cyclopentenolone, 2-methoxyphenol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), and 4-vinyl-2-methoxyphenol (Table II). These compounds contributed to brothy, caramel-like, sour or sweaty, and clove-like or spicy notes. These attributes described aroma profile of the aroma extract when it was sniffed directly. By the nature of aroma extract preparation, direct solvent extraction might cause low recovery of highly volatile compounds since they could be lost during sample preparation (*10*, *18*). With this reason, some compounds, *i.e.* dimethyl sulfide, ethyl propionate, and propanol could only be detected on DHS-GC-O (Table III).

AEDA is effective for screening of odor-active compounds; however this technique seemed to disregard matrix effects on volatile release (*6*). In reality, the odor is perceived when the odor-active compound is released from the food matrix and available for olfactory receptors. Since DHS exploits volatility difference of volatiles and non-volatile sample matrices (*10*), only the volatiles released and partitioned in a headspace are carried to GC. With this approach, DHS-GC-O enabled us to investigate release of volatile flavor compounds in the present study. However, it is important to remark that DHS is less sensitive to low or semi-volatile compounds, *e.g.* hydroxyl furanone and hydroxyl pyrone (*10*). To investigate the impact of individual volatile flavor compounds on the overall aroma profile, DHDA and odor activity value concept were employed. Odor activity value (OAV) was calculated based on ratio of concentration to odor threshold in water since water is the main matrix of soy sauce (*6*, *11*). However, this had to be remarked that other matrices of soy sauce, *i.e.* amino acids, peptides, carbohydrate, and brown pigment, as well as environmental conditions (pH and ionic strength) also played roles in flavor release.

From DHS-GC-MS, 27 volatile compounds were detected, whereas 18 of them were odor-active based on DHS-GC-O (Table III). From DHDA, 14 compounds were common to both soy sauce samples tested. 2-Methyl propanal, 1-propanol, 3-methyl-1-butanol, and methional were the most potent odor-active since they were perceived at the lowest minimal purge volume (Table III). According to the odor activity value concept, odorants contribute to the overall aroma if their concentrations exceed their odor threshold in a given matrix or OAV reaches the value of "1" (*19*). Considering OAVs, minimal purged volume from DHDA, and FD factor from AEDA of each odor-active compound, there were four occurrences of consensus and disagreement among them. Firstly, low volatile compounds, *i.e.* phenol and 4-ethyl phenol, had quite high FD value but were not odor-active in these two samples since their OAVs was less than 0.01. Secondly, 9 odorants perceived on DHDA agreed with OAVs which was higher than "1". Thirdly, some compounds, however had OAV less than "1", *i.e.* 1-propanol, 2,3-pentanedione, 1,6-dimethyl pyrazine, they were odor-active even at the lowest minimal purge volume. Fourthly, some compounds, such as 2-methyl propionic acid, butanoic acid, and methionol had high FD values and high OAVs (1.8-382.5), on the other hand, they were not detected on DHDA.

Moreover, different matrix effects of sample A and B were also observed in case of 2,3-butanedione and 3-hydroxy-2-butanone. Based on DHDA, these compounds had the OAVs in the range of 0.8-13.9 in both samples, but they were odor-active only in sample B. Moreover, some compounds, such as 2-methyl propanal, 2-methyl butanal, and 3-methyl butanal, had low minimal purge volume with high OAV (47.2-2424). They were perceived by the panelists at weak to medium intensity even at high purge volume. This was possibly due to the saturation of the odor-active compound to olfactory receptors. It has been discussed that OAVs should be used primarily to figure the impact of odor-active compounds. However, in some cases, OAV may not be appropriate for ranking of significance of those odor-active compounds in the matrix given (*20*).

From the results mentioned above, all odor-active compounds detected were classified into 5 groups based on their odor attributes; 1) malty, brothy, meaty, sulfury, 2) sweet, flowery, 3) caramel-like, vanilla-like, 4) sour, cheesy, sweaty, 5) clove-like, spicy, woody, 6) burnt, pyrazine-like, cooked rice-like, peanut-like. We concluded that 2-methyl propanal, 1-propanol, 3-methyl-1-butanol, 2,6-dimethyl pyrazine, mathional, 3-methyl butanoic acid, 2-methoxyphenol, 3-hydroxy-2-methyl-4H-pyran-4-one, and HDMF were important key aroma compounds of Thai soy sauce. Among these compounds, mathional, 3-methyl butanoic acid, and HDMF were also reported as key aroma compounds in Japanese soy sauce based on OAV concept (*6*). Moreover, 3-methyl-1-butanol, 2,6-dimethyl pyrazine, mathional, 2-methoxyphenol, and HDMF were mentioned to be common volatiles found in Chinese soy sauce (*9*).

Sensory Descriptive Analysis

Totally, 18 samples of Thai soy sauce were subjected to descriptive analysis and DHS-GC-MS. Firstly, we applied cluster analysis to cluster soy sauce samples tested based on their similarity of sensory attributes. There were 5 main groups of

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

samples. In addition, 15 sensory attributes of all samples were then explored by principal component analysis (PCA) aiming to extract the characteristic attributes of each group of Thai soy sauce. As a result, two principal components (PC) could be extracted from the original variables and could explain 57.16 % of the total variance (Figure 1). The sensory attributes contributed to the 1st PC were molasses-like aroma, alcohol aroma, fishy aroma, fishy flavor, and salty taste. This PC explained the variance of all variables up to 32.79 %. Sweet, sour, and bitter tastes were attributed to the 2nd PC which explained the variance at 24.37%. From graphical presentation (Figure 1), each group could be described for its character with the following attributes; group 1: sweet taste; group 2: fishy flavor, salty; group 3: sweet flavor; group 4: fishy aroma, group 5: soybean paste-like aroma and soybean paste-like flavor.

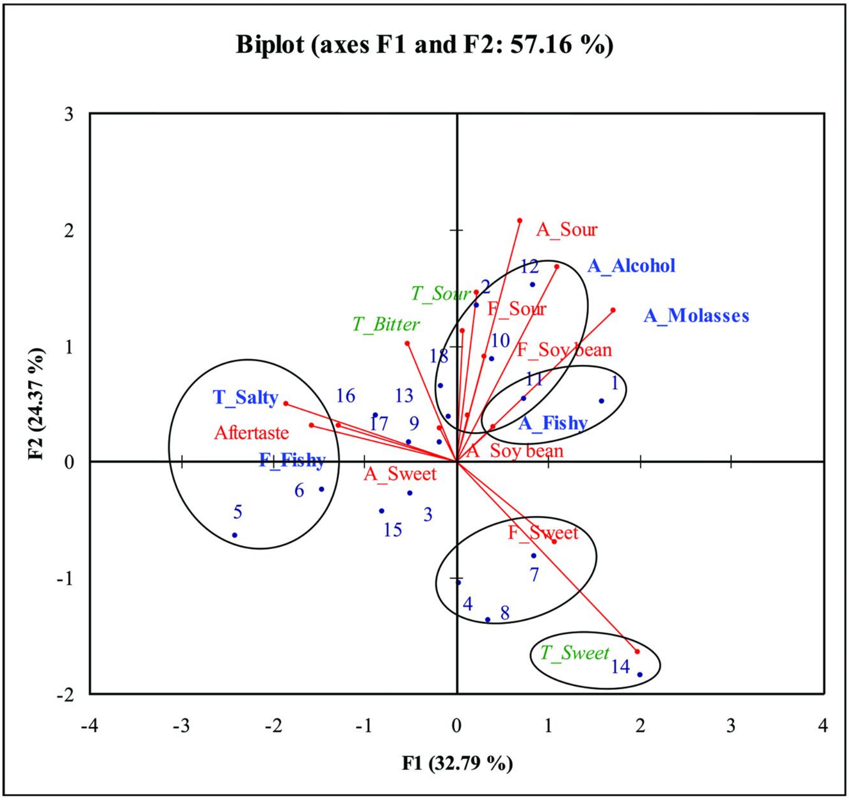


Figure 1. PCA biplot presenting relationships among soy sauce samples and sensory descriptive attributes obtained from 18 soy sauce samples. Sensory attributes with bold letter contributed to the 1st PC, while sensory attributes with italic letter contributed to the 2nd PC. The sensory attributes and samples displayed in the circles were correspondent to cluster analysis. A_aroma; F_flavor; and T_taste.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

In addition, correlations among sensory attributes and DHS volatile compounds were also observed (Table IV). As expected, sweet aroma attribute correlated with ethyl propionate which exhibited flowery odor. Sweet flavor attribute also correlated with the compound giving flowery, malty, and sweet odors. Sour aroma and sour sauce-like flavor were ascribed by acids. Molasses-like aroma was a complicated attribute showing correlation to varieties of compounds. Alcohol aroma had high correlation coefficient with molasses-like aroma (r = 0.74). Therefore volatile compounds correlated with this attribute were resembled to molasses-like aroma. Surprisingly, methionol which was not odor-active based on DHDA exhibited correlations with various attributes both for aroma and flavor. On the other hand, methional, 2,6-dimethyl pyrazine, and 2-methoxyphenol which were characterized as most potent odor-active compound in both AEDA and DHDA showed correlation to none of aroma and flavor attributes. We explained this occurrence as all samples contained these compounds at the level of substantial odor activity, so that they became saturated to panellists' perception.

Table IV. Pearson correlation coefficients among sensory attributes and DHS volatile compounds[a]

Attributes	*Aroma*					*Flavor*		
Compound	*Sweet*	*Molasses*	*Alcohol*	*Sour*	*Fishy*	*Sweet*	*Sour sauce*	*Soy bean paste*
Dimethyl sulfide		0.27	0.42	0.42		0.29		0.38
2-Methyl propanal		0.47				0.33		
2-Methyl butanal		0.48				0.36		
3-Methyl butanal	-0.28	0.30						-0.36
Ethyl propionate	0.29	0.32	0.48	0.35				0.33
1-Propanol			0.33					
2,3-Pentanedione						0.37		
2 and 3-Methyl-1-butanol		0.34			0.28			
Acetic acid		0.43	0.36	0.46			0.40	0.36
2 and 3-Methyl butanoic acid		0.33	0.39	0.53				0.31
Methionol		0.38	0.40	0.39				0.30

[a] Significance level at 95%.

The present study demonstrated the relationships among volatile compounds, volatile flavor compounds, and flavor profiles of Thai soy sauce through GC-O experiments, odor activity, and sensory analysis. The roles of key aroma compounds were also explained by those relationships.

Acknowledgments

This research project was granted by National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand (Grant No. BT-B-06-FG-14-4401).

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

References

1. Yokotsuka, T. Soy sauce biochemistry. *Adv. Food Res.* **1986**, *30*, 195–329.
2. Nunomura, N.; Sasaki, M. The Shelf Life of Soy Sauce. In *Shelf Life Studies of Foods and Beverages: Chemical, Biological, Physical and Nutritional Aspects*; Charalambous, G., Ed.; Elsevier Science Publisher B.V.: New York, 1993; pp 391−408.
3. Mongkolwai, T.; Assavanig, A.; Amnajsongsiri, C.; Flegel, T. W.; Bhumiratana, A. Technology transfer to small fermentation factories in Thailand: a consortium approach. *Food Res. Int.* **1997**, *30*, 555–563.
4. Valyasevi, R.; Rolle, R. S. An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *Int. J. Food Microbiol.* **2002**, *75*, 231–239.
5. Kobayashi, A.; Sugawara, E. Flavor Components of *Shoyu* and Miso Japanese Fermented Soybean Seasonings. In *Flavor Chemistry of Ethnic Foods*; Shahidi, F., Ho, C. T., Eds.; Plenum Press: New York, 1999; pp 5−14.
6. Steinhaus, P.; Schieberle, P. Characterization of the key aroma compounds in soy sauce using approaches of molecular sensory science. *J. Agric. Food Chem.* **2007**, *55*, 6262–6269.
7. Lee, S. M.; Seo, B. C.; Kim, Y. S. Volatile compounds in fermented and acid-hydrolyzed soy sauces. *J. Food Sci.* **2006**, *71*, 146–156.
8. Apriyantono, A.; Husain, H.; Lie, L.; Jodoamidjojo, M.; Puspitasari-Nienaber, N. L. Flavor Characteristics of Indonesian Soy Sauce (*Kecap manis*). In *Flavor Chemistry of Ethnic Foods*; Shahidi, F., Ho, C. T., Eds.; Plenum Press: New York, 1999; pp 15−31.
9. Zhang, Y.; Tao, W. Flavor and taste compounds analysis in Chinese solid fermented soy sauce. *African J. Biotechnol.* **2009**, *8*, 673–681.
10. Wanakhachornkrai, P.; Lertsiri, S. Comparison of determination method for volatile compounds in Thai soy sauce. *Food Chem.* **2003**, *83*, 619–629.
11. Acree, T. E.; Barnard, J.; Cummingham, D. A procedure for the sensory analysis of gas chromatographic effluents. *Food Chem.* **1984**, *14*, 273–286.
12. Grosch, W. Evaluation of the key odourants of foods by dilution experiments, aroma models and omission. *Chem. Senses* **2001**, *26*, 533–545.

13. Rotsatchakul, P.; Chaiseri, S.; Cadwallader, K. R. Identification of characteristic aroma components of Thai fried chili paste. *J. Agric. Food Chem.* **2008**, *56*, 528–536.
14. Lee, G. H.; Suriyaphan, O.; Cadwallader, K. R. Aroma components of cooked tail meat of American lobster (*Homarus americanus*). *J. Agric. Food Chem.* **2001**, *49*, 4324–4332.
15. Fernandez-Garcia, E. Use of headspace sampling in the quantitative analysis of Artisanal Spanish cheese aroma. *J. Agric. Food Chem.* **1996**, *44*, 1833–1839.
16. Sidel, J. L.; Stone, H. The role of sensory evaluation in the food industry. *Food Quality Pref.* **1993**, *4*, 65–73.
17. Maeztu, L.; Andueza, S.; Ibanez, C.; de Pena, M. P.; Bello, J.; Cid, C. Multivariate methods for characterization and classification of espresso coffees from different botanical varieties and types of roast by foam, taste, and mouthfeel. *J. Agric. Food Chem.* **2001**, *49*, 4743–4747.
18. Parliment, T. H. Solvent Extraction and Distillation Techniques. In *Techniques for Analyzing Food Aroma*; Marsili, R., Ed.; Marcel Dekker: New York, 1997; pp 1−26.
19. Schieberle, P. Recent Developments in Methods for Analysis of Flavor Compounds and Their Precursors. In *Characterization of Foods: Emerging Methods*; Goankar, A., Ed.; Elsevier: Amsterdam, The Netherlands, 1995; pp 403−431 .
20. Rychlik, M.; Schieberle, P.; Grosch, W. *Compilation of Odor Thresholds, Odor Qualities and Retention Indices of Key Food Odorants*; German Research Center for Food Chemistry: Garching, Germany, 1998.

Downloaded by STANFORD UNIV GREEN LIBR on June 24, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch023

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Chapter 24

Identification of Flavor-Active Volatiles in Soy Protein Isolate via Gas Chromatography Olfactometry

Anthony J. Irwin,*,[1] John D. Everard,[2] and Robert J. Micketts[1]

[1]Solae, 4300 Duncan Ave., St. Louis, MO 63110, USA
[2]DuPont Crop Genetics, Wilmington, DE 19880-0353, USA
*airwin@solae.com

Stir bar sorption and dynamic headspace purge techniques were used to recover the flavor volatiles from soy protein isolate and defatted soy flakes. Thirty-seven compounds were found to be the most flavor-active volatiles present in soy isolate by combining the results of both techniques. The majority of the volatiles are products of lipid oxidation, combined with a smaller number of amino acid degradation products. None of the individual components has a soy isolate flavor which is concluded to be a composite of them all. The full complement of flavor-active volatiles identified in soy protein isolate is also present in the defatted flake and a portion of these are carried through the isolate manufacturing process into the final product. Additionally, a significant amount of lipid remains bound to the soy protein and its continuing oxidation acts as a source of flavor volatiles throughout the manufacturing process. The flavor of the final product appears to be a composite of these two sources of volatiles.

Introduction

Soy products such as soy protein isolate provide a protein source that is less energy intensive and more environmentally friendly to produce than animal-based protein, while being at least as nutritious. However, its consumption by western consumers is limited by its characteristic flavor. These grainy, legumey off-flavors have been investigated and reported on for over 30 years. More than 300 volatile

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

organic compounds with a potential to impact the flavor have been identified in soy products (*1*). The identification and minimization of these constituents have been ongoing objectives for many soy protein producers.

While hundreds of volatiles can be identified in many foods, it is usually a much smaller number that contributes most of the flavor. These compounds are best identified by Aroma Extract Dilution Analysis (AEDA) (*2*) and similar techniques relying on gas chromatography olfactometry. Boatright (*3*) used a vacuum distillation technique and AEDA to show that the most powerful odorants in soy protein isolate were, in order of flavor intensity, dimethyl trisulfide, E,E-2,4-decadienal, an unknown, 2-pentyl pyridine, E,E-2,4-nonadienal, hexanal, an unknown, and acetophenone. Kobayashi (*4*) coupled an unspecified sample preparation technique with AEDA and found that E,E-2,4-nonadienal and E,E-2,4-decadienal were the strongest odors present in soymilk, followed by hexanal, 2-pentyl furan, 1-octen-3-one, hexanol, E-2-nonenal, and E,Z-2,4-decadienal. Acree (*5*) isolated flavor volatile fractions from soymilk using Freon and ethyl acetate extraction and used GCO to demonstrate that E,E-2,4-decadienal, hexanal, beta damascenone, E-2-nonenal, E-4,5-epoxy-(E)-2-decenal, vanillin, E,Z-2,6-nonadienal, E,E-2,4-nonadienal, and E,Z-2,4-decadienal were the most powerful flavor volatiles present. Since most of the flavor–active volatiles were found to be lipid oxidation products, lipoxygenase-free soy varieties have been developed in an attempt to reduce the intensity of the characteristic flavor of soy products. In our experience, these soy varieties have not yielded isolates with improved flavor characteristics.

Since no one isolation technique is likely to yield an extract that is totally representative of the volatile profile in the food being extracted, two techniques were selected to provide a more wide-ranging extraction of the flavor volatiles present in commercial and bench scale soy protein isolates. Firstly, stir bar absorptive technology (*6*) was selected because it is so convenient and because it was expected to recover the more hydrophobic, higher-boiling flavor volatiles. Secondly, the dynamic headspace purge and trap technique was selected because it was expected to preferentially isolate the more volatile flavors.

Materials and Methods

Extraction of Soy Isolate Flavor Volatiles via Stir Bar Sorption

Soy isolate (5g) was slowly added with stirring to a Waring blender containing deionized water (95ml). The slurry was stirred for 30 sec and then transferred to a sterile Nalgene container that was refrigerated overnight at 4C. Aliquots of slurry (20ml) were placed into 20mL Trace-Clean vials, fitted with Teflon-lined caps (VWR catalog #89093-834). A single Twister™ stir bar (10mm long X 0.5mm film thickness; Gerstel Inc., Baltimore, MD) was placed into each vial and the samples were stirred at 1700 rpm for 4 hr, at room temperature. At the end of the sampling period the slurries were discarded and the Twister™ bars were rinsed with seven exchanges of deionized water. The Twister™ bars were patted dry on Kimwipes, loaded into a Gerstel sample tubes and analyzed by GCMS. Because

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

of known losses of compounds from the volatile-loaded bars during dry storage, samples were prepared just prior to each olfactometry run.

Extraction of Defatted Soy Flake via Stir Bar Sorption

One Twister™ bar was stirred for 4 hr in a 7% aqueous slurry of commercial defatted flake from our production facility. The protein concentration of this slurry approximated that in a 5% soy isolate slurry.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

GCMS and GCO Analysis

The bars were loaded into a Gerstel TDS2 desorber unit that was operated in splitless/solvent vent mode. The bar was heated at a rate of 60C/min from 20C to 300C and then held for 3 min at 300C. The carrier gas flow during the desorption phase was 50ml/min. The desorbed volatiles were condensed at -150C in a CIS-4 inlet fitted with a liner filled with deactivated quartz wool and subsequently volatilized on to the GC column by heating to 300C at a rate of 12C/sec.

Gas chromatography was performed on a 50m 0.32mm Ultra 1 column with a 0.52micron film thickness mounted in an Agilent 6890. The initial oven temp of 40C was held for 6 min and the temperature was then increased at a rate of 5C/min until 270C was reached. The ramp rate was then increased to 20C/min until 325C was reached. Initial column flow rate was 2.4ml/min.

The volatiles exiting from the column were split between the Agilent 5973 MSD and the Gerstel Odor Detection Port (ODP) in a 1:4 ratio by a split union linking a Restek Hydroguard FS 0.18mm ID capillary column (1.322m) to the ODP and a Restek Hydroguard FS 0.10 mm ID capillary column (1.222m) to the MSD. The pressure at the split union was set to 2.2psi. Air makeup gas flow to ODP was 10ml/min with humidification. Mass spectral data was acquired over a 10 – 330 mass range.

Retention indices for compounds of interest were calculated from the retention times of ethyl ester standards according to equation 1.

$$RT_i = 100 \text{ x } (n + (\log(t_I) - \log(t_n))/(\log(t_{n+1}) - (\log(t_n))) \qquad (1)$$

where:

RT_i = Retention Time Index

n = Carbon number of n-paraffin or ester
(do not count carbons in ethyl group of ester)

t_i = Retention time of component (minutes).

t_n = Retention time of preceding standard (minutes).

t_{n+1} = Retention time of next n-standard (minutes)

In order to identify as many of the volatiles as possible, the total volatile load from eight stir bars was analyzed by GCMS analysis without splitting. Literature retention indices and, where available, authentic standards were used to confirm identifications.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

GCO Analysis of Stir Bar Volatiles

Aroma Extract Dilution Analysis (AEDA) was achieved by presenting 8X, 1X, 1/4, 1/16, and 1/64th of the amount volatiles collected on a single Twister™ bar to the GC column. This was accomplished either by loading multiple bars (for the 1X and 8X bar samples) or by splitting a portion of a single Twister™ bar extract during sample loading, so that only a defined portion (i.e., 1/4, 1/16, and 1/64th of the volatiles captured by a single bar) was presented to the column. The latter was achieved by pneumatically splitting portions of the sample, using the split/splitless capabilities of the Agilent/Gerstel 6890GC inlet, prior to the sample being introduced onto the chromatographic column. Preliminary experiments, in which defined ester mixtures were sequentially diluted (using the method described above) and quantified using the mass spectrometer, showed that proportional dilution using a combination of the number of bars loaded and the inlet pneumatics was effective and predictable.

Each AEDA analysis was performed in duplicate by authors JE and AI. Odor descriptors for both sniffers were compared and only those odors which were reproduced are reported in the final tabulation. Odors were considered as reproduced if (i) both panelists detected the odor at the same retention time in the same sample, or (ii) one of the panelists detected the same odor at the same retention time in other dilutions of the same sample.

Odors which were reproducibly detected during the 1X, 1/4, 1/16 and 1/64th bar experiments were thus detected at 1/8, 1/32, 1/128 and 1/512th respectively of the amount detected on eight bars. These odor compounds were assigned Flavor Dilution factors of eight, 32, 128 and 512 respectively.

Isolation of Flavor Volatiles via Dynamic Headspace Purge

A headspace purge system was assembled as shown in Fig 1.

The collection tube (Gerstel GC 09948) was packed with 290mg of 60/80 mesh Tenax-GR sorbent. Soy protein (5g) was stirred for 1 minute into 95ml deionized water in a Waring blender. The slurry was then sealed in an amber bottle and refrigerated overnight. A slurry sample (20g) was placed in the 50ml Erlenmeyer flask along with 7.5g sodium chloride and a magnetic stir bar. A teflon purge head adapter (SIS part 164372) was fitted to the Erlenmeyer flask, with a tube style purge head (SIS part 783009) fitted to the adapter. The sparging needle tip was positioned 3mm above the surface of the slurry. The assembly was then placed in a water jacketed beaker containing water at the level of the slurry in the flask and the water jacket was maintained at 45C. The nitrogen lines were attached and the slurry was stirred at 200rpm. The dry purge nitrogen stream was passed through the collection tube at 51ml/min to minimize water retention. The nitrogen flow rate through the sparge needle was 50ml/min. The collection tube was removed after 45 min purging.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

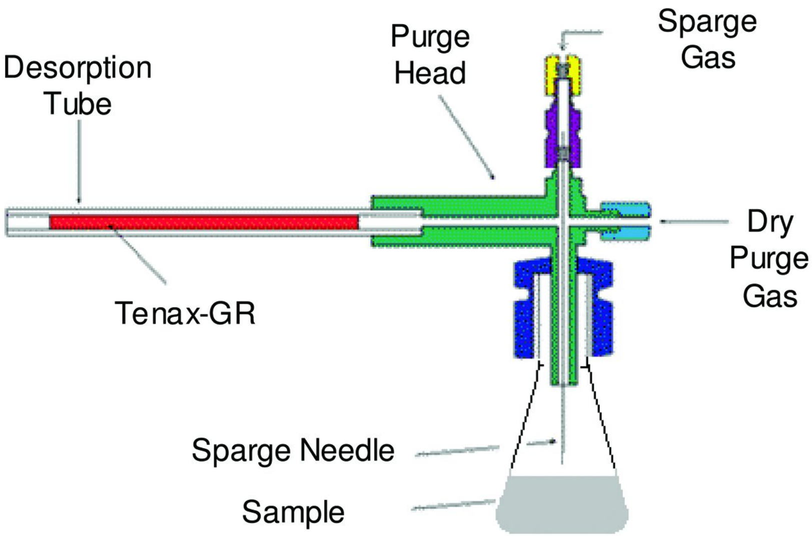


Figure 1. Dynamic headspace purge apparatus.

GCO Analysis of Headspace Purge Volatiles

The adsorbed volatiles were desorbed and analyzed by the same system described above. Dilutions of 1, ¼ and 1/16th of the total volatile load per collection tube were achieved as above via the pneumatic control system of the GC injector. Odors detected at ¼ and 1/16th dilutions were assigned FD factors of four and 16 respectively.

For standard mass spectral analysis, the total load of desorbed volatiles from one Tenax trap was chromatographed and transferred from the GC column to the mass spectral detector without splitting. Retention time indices were calculated for the two different instrument configurations (with and without noseport) and enabled accurate alignment of the odors and mass spectra.

Results and Discussion

GCO Analysis of Volatiles Recovered by Twister

A standard commercial isolate Supro ® 500E was selected as the first candidate for AEDA GCO analysis. When its volatiles were trapped on eight Twister™ bars and chromatographed without any splitting, 46 volatiles were detected reproducibly by one or both of the sniffers. The intensity of many of the odors was very high and it allowed the total load to be diluted stepwise by a factor of 128 (i.e. 1/16th of the total load of eight bars), at which point 20 volatiles could still be detected. These constituted the most odor-active volatiles in Supro ® 500E. Many of these compounds were subsequently identified by matching their RTI and mass spectra with those of authentic standards. Many unknowns were present that either had spectra which were not matched with any spectra in the NIST mass spectral library (version 2.0a), or were not present in sufficient quantity to generate a mass spectrum. The identities, RTIs, odor descriptors and flavor dilution (FD) factors of the volatiles detected in Supro ® 500E are listed in Table 1. Note that no odors with a retention index lower than that of hexanal were recovered by the stir bar sorption technique. Only when the instrument was configured without splitting for the noseport could volatiles be detected in this area of the mass spectral chromatogram.

Supro ® XT219D, a commercial isolate that is slightly hydrolyzed by an endo protease enzyme to improve its functionality, was analyzed by the same technique. At a 1/16th dilution of the eight bars (FD factor of 128), 20 volatiles were detected and their identities coincided with those found in the Supro ® 500E. In order to identify the most flavor-active compounds within this group, the AEDA dilution was taken to 1/64th of the eight bar extract. At this dilution, only eight volatiles were reproducibly detected. These included an unknown at RTI 510, 1-octen-3-one, an unknown at RTI 695, E-2-nonenal, E,E-2,4-nonadienal, E,Z-2,4-decadienal, E,E-2,4-decadienal, and 2-butyl-2-octenal. These eight compounds with a FD factor of 512 are the most odor-active compounds in this particular soy isolate, and probably Supro ® 500E as well. The unidentified odor at RTI 510 is almost certainly due to an overlap of two compounds since its descriptors varied with the isolate type and with the dilution factor (see details in Table 1.) Judging by their odors, the two volatiles appear to be a sulfur-containing compound and an aldehyde. The presence of both compounds simultaneously at the odor port gave a cracker-type odor.

A number of competitor commercial isolates, as well as isolates prepared in our bench scale pilot plant, were also analyzed by the same technique (results not shown). These were found to contain a similar range of volatiles as shown in Table 1, with similar FD factors.

None of the individual volatiles recovered by the Twister™ bar technique had a characterizing soy isolate odor.

GCO Analysis of Volatiles Recovered by DHS

The same Supro ® 500E-type isolate described above was also analyzed by using a DHS purge technique into a trap containing Tenax GR. Tenax TA was also evaluated as adsorbent and it was found to yield the same volatiles recovered by Tenax GR. The desorbed volatiles were split at 1:4 and 1:16 for AEDA analysis. The identities, RTIs, odor descriptors and flavor dilution (FD) factors of the volatiles detected in Supro ® 500E by DHS are listed in Table 2.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Table 1. All Odor Volatiles in Supro ® 500E, Supro ® XT219D and Defatted Soy Flake as Recovered by the Stir Bar Sorption Technique

RTI	*Identification*	*Descriptor*	*500E FD Factor*	*XT219D FD Factor*	*DFF Odor Intensity*
388-392	**Hexanal**	grass, green	**128**	**128**	**V. Strong**
444	Unknown 844	aldehyde varnish	ND	ND	Mod
466-468	**Unknown 468**	sulfur, boiled milk	**128**	**128**	**Mod**
481-483	**Methional**	boiloed potato	**32**	**128**	**Strong**
490	Unknown 490	putty, varnish	8	32	Strong
494	Heptanal	fruity	8	ND	Mod
509-512	Unknown 510A	sulfur, thiol		32	Mod
	Unknown 510B	paper aldehyde		**512**	**ND**
	Combination of 510A,B	cracker	32		
520-522	Unknown 521	sulfur, burning tires	8	8	ND
525	Unknown 525	sulfur, skunky	ND	ND	Mod
534	Unknown 534	sulfur	8	ND	Weak
560	Dimethyl trisulfide	sweaty socks, sulfide	8	32	ND
574-577	**1-Octen-3-one**	rotting wood, mushroom	**128**	**512**	**V. Strong**
579-580	Unknown 579	varnish, floral, mushroom	8	32	ND
584	1-Octen-3-ol	earthy, fruity	ND	ND	Strong
597-598	**Octanal**	fruity, orange	**128**	**8**	**Strong**
615-616	**Unknown 616**	cracker, paper	**128**	**128**	**Mod**
619-620	Unknown 620	varnish, tea	ND	ND	Strong
626-628	Unknown 628	sulfur, earthy, varnish	8	32	ND
634-637	**3-Octen-2-one**	grassy, varnish	**128**	**128**	**Mod**
651-653	2-Octenal	paper, varnish	8	32	Strong
668-670	**Unknown 669**	cooked meat, burnt	**128**	**128**	**Mod**
677-678	Unknown 677	mushroom, earthy	32	32	Mod
679-681	Unknown 680	paper, grass	32	32	Mod
685-686	E,E-3,5-Octadiene-2-one	coconut	8	8	ND
690	2-Nonanone	grassy, melon	ND	ND	Strong
693	Unknown 693	varnish		ND	Mod
693	Maltol	candy floss	32	32	ND
694-695	**Unknown 695**	stink bug	**128**	**512**	**Strong**
701-702	**Nonanal**	floral, fruity	**128**	**8**	**ND**
720-722	**2-Nonen-4-one**	stink bug, hay	**128**	**128**	**Strong**
736-738	**Unknown 737**	earthy, floral, grass	**32**	**128**	**Mod**
741-743	**E,E-2,6-Nonadienal**	paper, varnish	**32**	**128**	**Strong**
744-745	**E,Z-2,6 Nonadienal**	cucumber, varnish	**128**	**32**	**V. Strong**
753-755	**E-2-Nonenal**	cucumber, paper	**128**	**512**	**V. Strong**
767	Pentyl thiophene	grassy sulfur	ND	8	Weak
786-788	E,Z-2,4-Nonadienal	aldehyde, varnish	32	32	ND
798-800	**Unknown 798**	soapy aldehyde, putty[a]	**128**	**128**	**Strong**
803-806	**E,E-2,4-Nonadienal**	paper, soapy,	**128**	**512**	**V. strong**
813	Unknown 813	varnish,coconut	32	32	ND
823	Unknown 823	mushroom	8	8	ND
824-825	Unknown 824	stale hay	8	32	Mod
831-834	**3-Decen-2-one**	grassy, ethereal	**128**	**32**	**Mod**
863	Unknown 863	burning rubber	ND	ND	Strong
880-882	Unknown 880	floral, lily of the valley	8	32	ND
886	**E,Z-2,4-Decadienal**	soapy, paper	**128**	**512**	**Strong**
900-901	Unknown 901	roses	8	32	ND
905-907	**E,E-2,4-Decadienal**	paper, varnish	**128**	**512**	**V. Strong**
927	Unknown 927	floral varnish, paper	ND	ND	Mod
975-977	**2-Butyl 2-octenal**	paper[a]	**128**	**512**	**V. Strong**
979-981	**Damascenone**	fruity, hay, tea	**128**	**128**	**Strong**
1026-1028	Unknown 1026	tea, oversteeped	8	8	ND
1076-77	**Unknown 1077**	roses	**128**	**128**	**Strong**
1132	Unknown 1132	hay	8	ND	ND

[a] detected by JE only

ND = Not detected

Table 2. All Flavor-Active Volatiles Detected in Supro ® 500E by the DHS Technique

RTI	*Identity*	*Descriptor*	*FD Factor*
4.28[a]	Acetaldehyde	alcoholic	16
5.12[a]	Acetone/Propanal	alcoholic	16
6.96[a]	Diacetyl	caramel	16
234	3-Methyl butanal	malty	16
276	Pentanal	green	16
356	Unknown aldehyde	green	16
369	Unknown sulfur compound	sulfury	4
393	Hexanal	grass	16
404	Unknown	clammy	16
449	Unknown aldehyde	paper	16
485	Methional	boiled potato	16
493	Unknown aldehyde	paper	4
497	Heptanal	orange	16
513	Unknown	cracker	16
551	2-Heptenal	paper	16
561	Dimethyl trisulfide	sulfur stench	16
577	1-Octen-3-one	rotting wood	16
600	Octanal	orange	4
619	Unknown	cracker	4
630	Unknown aldehyde	papery	4
655	2-Octenal	varnish	16
679	Unknown aldehyde	paper	4
683	E,E-3,5-Octadiene-2-one	fruity	4
697	Unknown aldehyde	paper	16
704	Nonanal	fruity	4
747	E,Z-2,6-Nonadienal	varnish	4
759	E-2-Nonenal	paper	4
797	Unknown aldehyde	varnish	16
802	E,E-2,4-Nonadienal	paper	4
815	Unknown aldehyde	varnish	16
847	Unknown aldehyde	paper,fruity	16
888	E,Z-2,4-decadienal	paper	4
926	Unknown aldehyde	varnish	16
979	2-Butyl 2-octenal	paper	16
984	Damascenone	fruity tea	4

[a] Retention time in minutes since ethyl ester RTI calibration did not extend below 7.0 min.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Unlike the volatile profile recovered by the Twister™ bars that did not contain any detectable volatiles with a retention time lower than hexanal, the DHS volatile profile covered the entire chromatogram. Since the recovery of volatiles by DHS and stir bar sorptive technology was expected to be both quantitatively and qualitatively different, there was no realistic means of quantitatively comparing the FD factors of the two procedures. Instead, dilution of the Tenax-trapped volatiles was only pursued until the twenty or so volatiles with the highest FD factor were present. Ultimately, 22 volatiles were detected when the volatiles split at a 1:16 ratio and these were considered to represent the most flavor-active volatiles as recovered by the headspace purge technique. These were then compared with the 20 most flavor- active volatiles found by the stir bar sorption technique, albeit with quantitatively different FD factors.

The DHS technique recovered many odor-active volatiles that were not recovered by the Twister™ bar, and vice versa. As expected, the headspace purge procedure recovered much higher levels of lower molecular weight, lower boiling compounds such as acetaldehyde, diacetyl, 3-methyl butanal and pentanal that were not detectable as odors in the Twister™ bar volatiles. The polydimethylsiloxane coating of the bars seems to have a limited affinity for the low boiling volatiles known to be present, such that they are readily displaced by the more hydrophobic, higher boiling volatiles. The DHS technique also suggests that dimethyl trisulfide is an important contributor to soy isolate. The Twister™ bar recovered only a small amount of dimethyl trisulfide which did not persist during the AEDA.

Whereas the Twister™ volatiles are overwhelmingly derived from lipid oxidation and convey papery and varnishy flavors, those recovered by the headspace purge convey a combination of caramel, grassy and sulfide notes in addition to a selection of the former flavors. None of the volatiles detected with the DHS technique had a characteristic soy isolate aroma.

The Origins of Soy Isolate Flavor Volatiles

The 20 volatiles with the highest FD factors from both techniques are combined in Table 3. Since none of these volatiles have an odor resembling soy isolate, it is concluded that the latter's characteristic beany flavor is due to a combination of all of them. Many of the volatiles could not be identified because their spectra were not present in the mass spectral library or because there was insufficient compound to generate a mass spectrum.

All of the unidentified volatiles with papery and varnishy odors are certainly aldehydes because their odors are very similar to the numerous saturated and unsaturated aliphatic aldehydes that were identified by comparison with authentic standards. All such aldehydes that occurred at retention times higher than pentanal are almost certainly arising from oxidative degradation of linoleic and linolenic fatty acids (*7*, *8*). The other major source of aldehydes in food is the Strecker degradation of amino acids and none of the latter will yield aliphatic aldehydes with more than five carbons. With this assumption, 25 of the 37 flavor volatiles listed in Table 3 appear to be derived via lipid oxidation. Strecker degradation of amino acids accounts for the acetaldehyde, 3-methyl butanal and methional.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Degradation of the methional is probably the source of the dimethyl trisulfide. Diacetyl may arise from both the Maillard reaction and microbial growth during the aqueous processing stages of the isolate manufacturing process.

Soy protein isolate is prepared from defatted soy flakes via sequential aqueous extraction, centrifugation, precipitation at low pH, centrifugation, pasteurization, vacuum stripping, and spray drying. Given that most of the flavor volatiles in isolate were found to be derived via lipid oxidation, it was of interest to determine if they are being created during the manufacturing process from the flake, or if they are already present in the flake as a result of the soy oil extraction process. A sample of commercial defatted flake was therefore extracted as a 7% slurry with one Twister™ bar and the total volatile load was analyzed without splitting. A 7% slurry was used, as this concentration provided a similar soy protein level to the 5% slurries of soy isolate analyzed above.

Of the 20 volatiles with the highest FD factor (via Twister™ bar), 19 were present in the defatted flake (see DFF in Table 1). While AEDA was not performed on the defatted flake, the intensity of these odors was overwhelmingly higher than those experienced when sniffing the volatiles recovered from the isolates with one Twister™ bar. Extraction of soy oil from the beans with hexane and the subsequent solvent removal steps thus lead to production and retention of lipid oxidation volatiles in the defatted flake. The extraction and washing steps in the current commercial isolate process are successful in lowering the concentration of most of these volatiles. For example, 1-octen-3-ol is present at very high concentrations in defatted flake and it provides a strong odor impact during Twister™ GCO. However, it was not detected (by odor) in the isolate by either volatile recovery technique.

This behavior was not mimicked by the other volatiles, most of which retained sufficient concentration in the isolate to contribute to its characteristic flavor. For example, 1-octen-3-one remained as one of the most significant contributors to soy isolate flavor, despite having physical properties similar to 1-octen-3-ol. The latter should have led to removal of 1-octen-3-one in the washing and vacuumizing steps. The assumed removal of 1-octen-3-one in the washing/vacuumization steps and its probable subsequent regeneration in the pasteurization/drying steps can be traced to the residual fat in commercial defatted flake. Typically, the latter contains about 3% fat by acid hydrolysis, much of which is comprised of phospholipid. The isolate manufacturing process does not separate this residual fat from soy isolate, which consequently contains 4 – 5% fat by acid hydrolysis. This lipid accompanying the protein through the isolate manufacturing process appears to serve as a continuous source of lipid oxidation volatiles whose regeneration in thermal steps competes with their removal during washing/vacuumization. Overall, the net result of the isolate manufacturing process is a reduction of the volatile levels present in defatted flake, despite the continued presence of residual fat.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Table 3. Volatiles with the Highest FD Factors in Supro ® 500E Soy Protein Isolate as Detected via the DHS and Stir Bar Sorption Techniques

RTI/RT	*Compound*	*Detected at 1/16 DHS tube*	*Detected at 1/16 Twister bar*	*Probable Origin*
4.28 min	Acetaldehyde	Yes	No	Alanine -Strecker degradation
6.96 min	Diacetyl	Yes	No	Maillard
234	3-Methyl butanal	Yes	No	Leucine-Strecker degradation
276	Pentanal	Yes	No	Lipid oxidation
356	Unknown aldehyde	Yes	No	Lipid oxidation
393	Hexanal	Yes	Yes	Lipid oxidation
406	Unknown	Yes	No	Unknown
449	Unknown aldehyde	Yes	No	Lipid oxidation
468	Unknown	No	Yes	Unknown
482	Methional	Yes	Yes	Methionine -Strecker degradation
497	Heptanal	Yes	No	Lipid oxidation
512	Unknown	Yes	No	Unknown
551	2-Heptenal	Yes	No	Lipid oxidation
561	Dimethyl trisulfide	Yes	No	Methional
577	1-Octen-3-one	Yes	Yes	Lipid oxidation
600	Octanal	No	Yes	Lipid oxidation
615	Unknown	No	Yes	Unknown
636	3-Octen-2-one	No	Yes	Lipid oxidation
655	2-Octenal	Yes	No	Lipid oxidation
669	Unknown	No	Yes	Unknown
695	Unknown aldehyde	Yes	Yes	Lipid oxidation
704	Nonanal	No	Yes	Lipid oxidation
721	2-Nonene-4-one	No	Yes	Lipid oxidation
742	E,E-2,6-Nonadienal	No	Yes	Lipid oxidation
744	E,Z-2,6-Nonadienal	No	Yes	Lipid oxidation
754	E-2-Nonenal	No	Yes	Lipid oxidation
798	Unknown aldehyde	Yes	Yes	Lipid oxidation
804	E,E-2,4-Nonadienal	No	Yes	Lipid oxidation
813	Unknown aldehyde	Yes	No	Lipid oxidation
832	3-Decen-2-one	No	Yes	Lipid oxidation
843	Unknown aldehyde	Yes	No	Lipid oxidation
886	E,Z-2,4-Decadienal	No	Yes	Lipid oxidation
905	E,E-2,4-Decadienal	No	Yes	Lipid oxidation
926	Unknown aldehyde	Yes	No	Lipid oxidation
977	2-Butyl 2-octenal	Yes	Yes	Lipid oxidation
980	beta Damascenone	No	Yes	Carotenoid oxidation
1077	Unknown	No	Yes	Unknown

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Conclusion

The presence of the characterizing soy isolate flavors in the defatted flake strongly suggests that processing attempts to minimize the off-flavor of soy isolate during its manufacturing from the current raw material will be problematic. Efforts to produce a bland-tasting soy isolate should be focused either on genetically-modified beans with reduced polyunsaturated fatty acid content, or on conventional beans whose protein is separated from the oil in a novel way that minimizes exposure of the soy protein to oxidizing triglycerides and phospholipids.

References

1. MacLeod, G.; Ames, J. Soy flavor and its improvement. *CRC Crit. Rev. Food Sci. Nutr.* **1988**, *27*, 219−400.
2. Grosch, W. Determination of potent odourants in foods by aroma extract dilution analysis (AEDA) and calculation of odour activity values (OAVs). *Flavour Fragrance J.* **1994**, *9*, 147–158.
3. Boatwright, W. L.; Lei, Q. Compounds Contributing to the beany odor of aqueous solutions of soy protein isolates. *J. Food Sci.* **1999**, *64*, 667–670.
4. Kobayashi, A.; Tsuda, Y.; Hirata, N.; Kubota, K.; Kitamura, K. *J. Agric. Food Chem.* **1995**, *43*, 2449–2452.
5. Feng, Y.; Acree, T. E.; Lavin E. H. Processing Modulation of Soymilk Flavor Chemistry. In *Aroma Active Compounds in Food*; ACS Symposium Series 794; Takeaka, G. R., Guntert, M., Engel, K.-H., Eds.; 2001; pp 251−264.
6. Baltussen, E.; Sandra, P.; David, F.; Cramers, C. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: Theory and principles. *J. Microcolumn Sep.* **1999**, *11*, 737–747.
7. Whitfield, F. B. Volatiles from interactions of Maillard reactions and lipids. *Crit. Rev. Food Sci. Nutr.* **1992**, *31*, 1–58.
8. Grosch, W. Reactions of Hydroperoxides: Products of Low Molecular Weight. In *Autoxidation of Unsaturated Lipids*; Chan, H. W.-S., Ed.; Academic Press: London 1987; pp 95−139.

Downloaded by OHIO STATE UNIV LIBRARIES on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ch024

Subject Index

A

- ABTS. *See* 2, 2′-Azinobis (3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt
- Aglycone, 94
  - isoflavone, 203*f*, 209, 210, 210*t*, 211*t*
- All soy, all sugar syrup bar formulation, 297, 298*t*, 311*f*, 317*f*
- β-Amyrin, 97*f*, 98*f*
- Anti-oleosin antiserum, 263*f*
- Antioxidant activities, soy flour extract, defatted, 201
- Aroma components and soymilks, 361, 366*t*
- Aw. *See* Water activity
- Ayakogane, 266*f*
- 2, 2′-Azinobis (3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt, radical scavenging activity, 159, 163*f*

B

- BBI. *See* Bowman-Birk inhibitor
- BC. *See* β-Conglycinin
- Bile acid, 54*t*, 55*t*
- Blanching
  - soybeans, 27, 33, 35*t*, 36, 37*t*, 38, 39*t*
  - soymilk, 26, 34*t*
- Bovine serum albumin, 108*f*, 110*f*
- Bowman-Birk inhibitor, 134, 144*t*
  - amino acid sequence, 150*f*
  - soybean, 135*f*
- BSA. *See* Bovine serum albumin

C

- $CaCl_2$. *See* Calcium chloride
- Calcium chloride
  - protein solubility, 223*f*
  - soybean oil body, 109*f*
  - soymilk, 267*t*
  - tofu, 125*t*
- Calcium lactate
  - oil release in emulsion, 45, 54*f*, 55*t*
  - o/w emulsions, 52*f*, 55*f*
  - protein
    - aqueous phase, 57*f*
    - cream phase, 54*f*, 57*f*
    - digestibility, 45
    - MW profiles, 57*f*
- Carbon dioxide and 7S and 11S soy proteins, 76
- Carbon-centered radicals
  - ISP, 10*f*, 11*f*, 12*f*, 14*f*, 15*f*
  - soy protein products, 3
- β-Carotene-linoleic acid method, 160, 164*f*
- Casein, 5*f*
- Caseinate, 45
- $CaSO_4 \cdot 2H_2O$ and tofu, 226*f*
- CBB G-250 staining, 265*t*
- Cereals, soy, 325, 326
- Chymotrypsinogen, 108*f*, 110*f*
- CLSM. *See* Confocal laser scanning microscopy
- Confocal laser scanning microscopy, 52*f*, 55*f*
- β-Conglycinin, 145
  - bioactive peptides/hydrolysates, 148*t*
  - and oil body, 106
  - soybean, 121*t*, 122*t*, 147*f*, 225*f*
  - storage effect, 120
- Consumer
  - extruded soy foods, acceptance, 323, 325
  - food bar, perception, 334*t*
- Crosshead speed and tofu hardness, 240*t*, 241*t*, 242*f*, 243*f*

D

- Daidzein conjugates, 182*f*
- Degree of hydrolysis, 306*f*, 307*f*
- DH. *See* Degree of hydrolysis
- DHA. *See* Docosahexaenoic acid
- DHS. *See* Dynamic headspace
- 2, 2-Diphenyl-1-picrylhydrazyl
  - radical scavenging activity, 159, 164*f*
- Docosahexaenoic acid, 205, 207, 208*f*, 212*f*, 213
- DPPH. *See* 2, 2-Diphenyl-1-picrylhydrazyl
- Dynamic headspace, 377
  - purge
    - apparatus, 393*f*
    - flavor volatiles, isolation, 392
  - technique, 396*t*, 399*t*
  - volatile compounds, 385*t*

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

E

- Egg albumin, 5*f*
- Eicosapentaenoic acid and menhaden oil, 207, 208*f*, 212*f*, 213
- Electron paramagnetic resonance spectroscopy
  - casein, 5*f*
  - egg albumin, 5*f*
  - ISP, 5*f*, 6*t*, 10*f*, 11*f*, 12*f*, 14*f*, 15*f*, 17*f*, 18*f*
  - rancid soybean oil, 5*f*
  - sodium caseinate, 5*f*
  - soy protein, 6*t*, 7*f*, 8*t*
  - soybean flour, 16*f*, 17*f*
  - whey protein isolate, 7*f*
- Emulsion
  - Ca lactate, 52*f*
  - oil release, 45
  - oil-in-water, 48
  - two-stage heating process, 54*f*, 55*f*
- Endopeptidase, 312*f*
- Enrei, 266*f*
- Enzyme and 7S and 11S soy protein separation, 78
- EPA. *See* Eicosapentaenoic acid
- EPR. *See* Electron paramagnetic resonance spectroscopy
- Extruded soy foods, 324
  - concept testing, 334
  - consumer acceptance, 323, 325
  - consumption, 323
  - marketing strategies, 334
  - snacks, 330*f*

F

- Fe^{2+}-chelating activity, 160, 165*f*
- Fermentation
  - soybeans
    - antioxidative activity, 155
    - components changes, 155
    - isoflavone, 162*f*
    - phytic acid, 163*f*
    - saponin, 163*f*
- Fish oil, 204, 205
  - oxidation, 206
  - soy flour extracts, defatted, 208
- Flavor
  - binding
    - capacities, 350
    - food proteins, 340
    - parameters, 348, 352*t*
  - compounds, 340
  - soy protein interactions, 339, 340
    - factors, 353
    - measurement techniques, 344, 347
  - Thai soy sauce, 375
  - volatile compound, 375
    - soy protein isolate, 389
- Food bar
  - consumer perception, 334*t*
  - formulations, 296
  - texture, 293
- Food proteins and flavor compounds binding, 340
- Free radicals and soy protein, 4, 9
- F_2 seed hypocotyls and saponin contents, 100*f*
- Fukuyutaka, 261*f*, 262*f*, 263*f*, 266*f*

G

- Gas chromatography-olfactometery, 364, 377, 382, 389, 391, 392, 393, 394
- GCO. *See* Gas chromatography-olfactometery
- GDL. *See* Glucono-1-5-lactone
- Genistein conjugates, 183*f*
- Glucono-1-5-lactone, tofu and soymilks phytate contents, 253*f*, 254
- Glucoside isoflavones, 203*f*, 209, 210, 210*t*, 211*t*
- *Glycine max* (L.) Merr., 91
- Glycinin
  - oil body, 106
  - soybean, 122*t*
  - storage effect, 120, 122*t*
- Glycitein conjugates, 184*f*
- Grinding methods and soymilks aroma components, 361, 366*t*
- Guo and 7S and 11S soy proteins separation, 81

H

- Hatayutaka, 266*f*
- Heating sequence
  - oil release in emulsions, 45, 52*f*, 54*t*
  - one-stage heating process, 52*f*
  - protein
    - content, 52*f*
    - digestibility, 45
    - MW profiles, 53*f*
  - two-stage heating process, 52*f*, 54*f*, 55*f*, 55*t*
- Hexanal
  - and menhaden oil, 210*f*

  and soymilk, 35*t*
Hydrolysis, types, 314*f*

I

IGC. *See* Inverse gas chromatography technique
India and soy foods product, 332
Industrialized production technology method, 7S and 11S soy proteins separation, 86
Instron textural profiles and tofu products, 231, 241*t*
Instron universal testing machine, 237*f*
International markets and soy foods product, 332
Inverse gas chromatography technique, 345
Isoflavone, 123, 158
  conjugates, interconversion, 182, 184*f*
  contents
    dry-thermal effects, 179, 180*t*
    fermentation of soybeans, 162*f*
    moist-thermal effect, 176*t*, 177, 178*t*
    soy flour extracts, 205
  conversion, 171
  HPLC analysis, 175
  sources, 189
  soy flour extracts, defatted, 211*f*, 212*t*
  soy foods, 195
  soy protein ingredients, 189, 193*t*, 194*f*
  soy seeds, 191
  soybean, 171
Isolated soy protein, 315*f*
  bar functionality, 309
  bulk density, 310*f*
  carbon-centered radicals, 10*f*, 11*f*, 12*f*, 14*f*, 15*f*
  EPR, 5*f*, 6*t*, 18*f*
  hydrolysis, 314*f*, 315*f*
  products, 311*f*
  Supro® 313, 296, 311*f*, 317*f*, 318*f*
  Supro® 320, 296, 311*f*, 317*f*
  Supro® 430, 315*f*, 317*f*, 318*f*
  Supro® 661, 311*f*
  Supro® 313/320 blend, 317*f*
  Supro® 320/313 blends, 311*f*
  Supro® 500E, 395*t*, 396*t*, 399*t*
ISP. *See* Isolated soy protein

K

KTI. *See* Kunitz trypsin inhibitor
Kunitz trypsin inhibitor, 137, 138*f*
  amino acid sequence, 150*f*

L

LC-MS/MS profile analysis, 91
LC/PDA/MS. *See* Tandem mass spectrometry
Ligand-protein binding, 349*t*
Lipoxygenase, 29
Lunasin
  amino acid sequence, 140*f*, 150*f*
  isolation and purification methods, 142*f*
  soybean, 140*f*, 144*t*
Lysozyme, 108*f*, 110*f*

M

Magnesium chloride
  and soymilk, 259, 261*f*, 262*f*, 263*f*
  and tofu, 257*f*
Menhaden oil
  soy flour extracts, defatted, 208*f*
    DHA and EPA, 207, 208*f*, 212*f*, 213
    hexanal production, 210*f*
    TBA reactive oxidation products, 208*f*
Mg-tofu, soymilks phytate contents, 253*f*
Microthermics UHT Processor, 38*f*
Mutation and soyasapogenol A-deficient soybean, 91

N

NaCl and soymilk, raw, 107*f*
Nagano method, 7S and 11S soy proteins, 74
Nama-shibori soymilk, 267*t*
  calcium, potassium, and magnesium, 272*t*
  calcium chloride, 267*t*
  okara, 273*t*
  particle size distribution, 268*f*
  SDS-PAGE patterns, 271*f*, 274*f*
  solids, distribution, 269*f*
Natto, 157
  peptide maps, 162*f*
  water extracts, activity, 165*f*

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

O

OAV. *See* Odor-activity values
OCC. *See* Optimal coagulant concentration
Odor-activity values, 381*t*
Oil body, soybean, 105
  and β-conglycinin, 106
  and glycinin, 106
  pH and $Cacl_2$, 106
  protein, interaction, 107
    heating effect, 110, 110*f*
    soymilk, 103, 104
  surface hydrophobicities, 106
Oil-in-water emulsion
  and Ca lactate
    one-stage heating process, 52*f*
    two-stage heating process, 52*f*, 55*f*
  preparation, 48
  protein digestion, 48
Okara, 260
  composition, 288*t*
  Nama-shibori soymilk, 273*t*
  SDS-PAGE patterns, 274*f*
  whole-soybean tofu, 284*t*
Optimal coagulant concentration, 125*t*
Ovalbumin, 108*f*
O/W. *See* Oil-in-water emulsion

P

Phytate
  soybean, 249, 250
  soymilk, 252*f*
  storage effect, 249
  tofu texture, 249
Phytic acid, 159
  fermentation of soybeans, 163*f*
Powdered soy protein, storage, 9
Protein
  bar system, 295*f*
  digestion
    calcium lactate, 45
    heating sequence, 45
    oil-in-water emulsion, 48
  functionality, 293
  hydrolysis, 161*f*
  precipitate, 265*t*
Protein, all soy, all sugar syrup bar formulation
  bar hardness, 305*f*, 317*f*
  DH vs. chewiness, 307*f*
  DH vs. mechanical bar hardness, 306*f*
  solubility vs. mechanical bar hardness, 305*f*
  Supro® 320/313 blends, 311*f*
Protein, soy, no sugar syrup formulation, 318*f*
Proto soybean, 32*t*
  storage, 124*f*, 126*f*
  tofu, 117*f*

R

Random hydrolysis and food protein, 314*f*

S

SA. *See* Soyasapogenol A
Sachiyutaka, 261*f*, 262*f*, 263*f*, 266*f*
SAFE. *See* Solvent-assisted flavor evaporation
7S and 11S soy proteins
  industry separation method, 71, 85, 85*t*
    Guo, 81
    sale, 79*t*
    scale, 82, 82*t*, 83*t*
    Wu and Ricket, 79
  laboratory separation methods, 71
    Ca^{2+}, 77
    CO_2, 76
    enzyme, 78
    Mg^{2+}, 77
    Na^+, 77
    Nagano, 74
    sale, 75*t*
    scale, 78, 82, 83*t*
    Thanh, 73
    Wu and the modified methods, 74
Saponin, 95, 158
  chemical structure, 93*f*
  F_2 seed hypocotyls, 100*f*
  fermentation of soybeans, 163*f*
  hydrolysis, 94
  Shirosennari and Sg-5 phenotypes, 94, 100*f*
  soybean seed hypocotyls, 94
SB. *See* Soyasapogenol B
SCN. *See* Sodium caseinate
SDS-PAGE. *See* Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Selective hydrolysis and food proteins, 314*f*
Semi-kanetsu-shibori soymilk, 267*t*, 268*f*, 269*f*, 271*f*, 272*t*, 273*t*, 274*f*
Sensory evaluation
  cereals, 325, 327*t*
  soy foods, extruded, 323

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

  soy snacks, 325
  tofu, 244, 288*t*
Shirosennari and Sg-5 phenotypes, 100*f*
Sodium caseinate, 5*f*
Sodium chloride and soymilk, 107*f*
Sodium dodecyl sulfate polyacrylamide gel electrophoresis, 49, 105, 157, 265*t*, 274*f*
SOD-like activity. *See* Superoxide dismutase-like activity
Solvent-assisted flavor evaporation, 363
Soyasapogenol A
  biosynthesis, 96*f*
  deficient mutant, 91
  HPLC patterns, 98*f*
  LC-MS/MS profile analysis, 91
  MS fragment patterns, 97*f*
  structure, 97*f*
Soyasapogenol B
  biosynthesis, 96*f*
  HPLC patterns, 98*f*
  MS fragment patterns, 97*f*
  structure, 97*f*
Soybean, 176*t*
  annual per capita consumption, 334*t*
  black, 174*f*
  blanching, 27, 35*t*, 36, 37*t*, 38
  Bowman-Birk inhibitor, 134, 135*f*
  color and biochemical changes, 113
  color indices, 128*t*
  components
    coagulation reactivity, 255
    soymilk, 255
    tofu texture, 255
  composition, 288*t*
  β-conglycinin, 121*t*, 145, 225*f*
  curd formation, 220
  daidzein conjugates, interconversion, 182*f*
  fermentation, 155
  flour, defatted, 16*f*, 17*f*
  glycinin, 122*t*, 225*f*
  IA2032, 32*t*
  isoflavones, 171, 175, 176*t*, 177, 178*t*, 179, 180*t*
  KS1, 176*t*, 180*t*
    daidzein conjugates, 182*f*
    genistein conjugates, 183*f*
    glycitein conjugates, 184*f*
  KS8, 178*t*, 180*t*
  Kunitz trypsin inhibitor, 137
  lunasin, 140*f*, 142*f*, 144*t*
  oil, rancid, 5*f*
  oil body, 105
    $CaCl_2$ effects, 109*f*
    heating effects, 108
    pH effects, 109*f*
    protein compositions, 108*f*
    surface hydrophobicities, 110*f*
  peptides
    chemistry and biological properties, 133
    isolation, purification and characterization, 149*f*
    and proteins, 133
  phytate content, 250, 251
    Mg tofu texture, 252
    and tofu texture, 249
  pretreated, 174*f*
  proto, 32*t*
  saponin components, 93*f*, 94
  storage, 113
    Aw, 125*t*
    $CaCl_2$, 125*t*
    color and tofu yield, 115
    isoflavones, 123
    phytate content, 118
    surface color, 116*t*
    titratable acidity, 115
    tofu yield and textural properties, 118*t*
  thermal treatments, 171
  TN3, 178*t*, 181*t*
  TN6, 178*t*, 181*t*
    daidzein conjugates, 182*f*
    genistein conjugates, 183*f*
    glycitein conjugates, 184*f*
  tofu analogue, 277, 280
  water activity, 125*t*
  whole, 277
  yellow proto variety, 116*t*
Soy flake, defatted and odor volatiles, 395*t*
Soy flour extracts, defatted
  antioxidant capabilities, 201
  enzymatic hydrolysis, 211*f*, 212*t*
  and fish oil, 208
  heat or enzyme treated, 204
  isoflavones, 205, 211*f*, 212*t*
  menhaden oil, 207, 208*f*
    DHA and EPA, 205, 207, 212*f*, 213
    hexanal production, 210*f*
  phenolic content, 205
  preparation, 204
Soy foods
  extrusion, 324
  health food, 323
  product
    development, 332
    India, 332
    international markets, 332
  sensory evaluation techniques, 323
  snacks, 324
Soy isoflavones, 194*f*, 195
Soy isolate flavor volatiles, 390, 397

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

- Soymilk
  - aroma components, 361, 366*t*
  - blanching, 26
  - calcium chloride, 267*t*
  - calcium effects, 223*f*
  - cold grind and hot grind
    - aroma component, 366*t*
    - methods, 361
    - odor-activity values, 368*t*
  - formation, 220, 222*f*
  - heated, 63, 66*f*, 222*f*
    - surface hydrophobicity, 66*f*, 69*f*
    - surface SH content, 65*f*, 68*f*
  - hexanal, 35*t*
  - lipids and proteins, 259
  - making, 113
  - making properties, 128*t*
  - $MgCl_2$, 259, 261*f*, 262*f*, 263*f*
  - mineral components, 260
  - NaCl effect, 105, 107*f*
  - oil body and protein, interaction, 103, 104
  - physichochemical properties, 266
  - phytate content, 252
    - GDL-tofu, 253*f*
    - Mg-tofu, 253*f*
  - precipitation, 67*f*
  - preparation, 63
  - products, 39, 40*t*
  - protein
    - composition, 219
    - cooling rate, 61
    - denaturation, 61, 64
    - particle content, 225*f*
    - solubility, 223*f*
    - surface hydrophobicity, 63
    - surface SH content, 63
  - refrigerated, 66*f*
  - SDS-PAGE patterns, 271*f*
  - and soybean components, 255
  - 11S/7S ratio and coagulation reactivity, 264
  - storage, 117*f*, 126*f*
  - tofu curd formation, 224*f*
  - traditional and steam-injection batch processes, 25
  - trypsin inhibitor activity, 23, 29, 32*t*, 36*f*, 39
    - direct-UHT processing methods, 37*t*
    - indirect-UHT processing methods, 39*t*
  - UHT methods, 26, 36, 38
  - Vinton soybeans, 126*f*
- Soy odor and hexanal, 28
- Soy protein, 296
  - flavor interactions, 339, 341, 342, 350, 352*t*
    - factors affecting, 353
  - functionality and food bar texture, 293, 303
  - ingredients
    - isoflavone, 189, 191, 193*t*, 194*f*
    - production, 193*f*
  - isolate, 7*f*
    - flavor-active volatiles, 389
  - mechanical bar hardness *vs.* sensory overall liking, 302*f*
  - powdered drink mixes, 6*t*
  - preheated, 45
  - products, 8*t*
    - carbon-centered radicals, 3
    - free radicals, 4
  - properties vs. bar functionality, 316*t*
  - radicals, 13*t*
  - 7S and 11S, 71
- Soy sauce, Thai, 375
  - aroma extract, 380*t*
  - odor-active compounds, 381*t*
  - sensory descriptive attributes, 384*f*
- Soy seeds and isoflavone, 191
- Soy snacks, 325, 326, 328*t*
- Steam-injection methods, 30, 30*t*
- Stir bar sorption
  - odor volatiles, 395*t*
  - soy isolate flavor volatiles, 390
  - techniques, 399*t*
- Storage and soybeans, 113
- Superoxide dismutase-like activity, 160, 165*f*
- Supro® 313, 296, 311*f*, 317*f*, 318*f*
- Supro® 320, 296, 311*f*, 317*f*
- Supro® 430, 315*f*, 317*f*, 318*f*
- Supro® 661, 311*f*
- Supro® 313/320 blend, 317*f*
- Supro® 320/313 blends, 311*f*
- Supro® 311 Nuggets, 296, 310*f*
- Supro® 500E, 395*t*, 396*t*, 399*t*
- Supro® Nuggets, 309*f*
- Supro® XT219D, 395*t*

T

- T-152
  - hydrolysate, 98*f*, 99*f*
  - sg-5 phenotypes, 100*f*
- Tandem mass spectrometry, 94
- TEAC. *See* Trolox equivalent antioxidant capacity
- Thai soy sauce
  - flavor profiles, 375, 378, 379*t*
  - volatile flavor compounds, 375

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002

Thanh and 7S and 11S soy proteins separation, 73
Thin layer chromatography, 95
TIA. *See* Trypsin inhibitor activity
TLC. *See* Thin layer chromatography
TNBS. *See* Trinitrobenzenesulfonic acid
Tofu
  analogue, 277
  breaking stress, 267*t*, 273*t*
  $CaSO_4 \cdot 2H_2O$, 226*f*
  composition, 288*t*
  conventional, 287*f*, 288*t*
  crosshead speed, 240*t*, 242*f*, 243*f*
  curd, 224*f*, 225*f*
  formation, 220
  Instron texture analysis, 231
  Japanese varieties, 266*f*
  layers, 238*f*
  making, 113, 125*t*, 233*f*
    $CaCl_2$, 125*t*
    conventional, 279
    proto, 127*f*
    storage effect, 123, 127*f*
    transglutaminase-treated, 280
    whole soybean, 280
  $MgCl_2$ and breaking stress, 257*f*
  network structure and coagulant, 227*f*
  no-skin, 242*f*, 243*f*
  particulate protein content, 267*t*, 273*t*
  preparation, 251
  products, 236*t*, 239*f*
    crosshead speed, 241*t*
    hardness, 245*t*, 246*t*
    Instron textural profiles, 241*t*
    plunger penetration, 241*t*, 244
    sensory scores, 245*t*, 246*t*
    springiness characteristics, 245*t*, 246*t*
  proto soybeans, 117*f*
  quality evaluation, 231
  soymilk and $CaSO_4 \cdot 2H_2O$, 226*f*
  11S/7S ratio and $MgCl_2$, 266*f*
  stainless cutter, 238*f*
  storage influence, 118*t*, 125
  structure
    coagulant concentration, 219, 221
    soymilk protein composition, 219
    11S/7S ratio, 221
  texture, 251
    phytate content, 249
    profile, 258
    soybean components, 255
  transglutaminase treated, 283, 284*t*, 285*t*
  water retention ability, 282
Tosan 205, 110*f*, 226*f*, 261*f*, 262*f*, 263*f*
Transglutaminase and tofu, 280, 283, 284*t*, 285*t*
Trinitrobenzenesulfonic acid method, 161*f*
Trolox equivalent antioxidant capacity, 163*f*, 164*f*
Trypsin inhibitor activity
  analysis, 28
  health benefits, 40
  and hexanal, 35*t*
  inactivation, 38*f*
  kinetic analysis, 29
  soymilk, 23, 29, 32*t*, 35*t*
    direct-UHT processing methods, 37*t*
    indirect-UHT processing methods, 39*t*
    products, 39, 40*t*
  soymilk, 36*f*
  traditional and steam-injection method, 30, 30*t*
  UHT methods, 31

U

UHT methods. *See* Ultra-high temperature methods
Ultra-high temperature methods, 32*t*
  soymilk, 26, 36, 38
  trypsin inhibitor activity, 27, 31, 32*t*, 37*t*, 38*f*

V

Vinton soybeans, 126*f*
Volatile flavor compounds
  soy protein isolate, 389
  Thai soy sauce, 375

W

Water activity and soybean, 125*t*
Water retention ability and tofu, 281, 284*t*, 286*t*
Water-to-bean ratios and tofu, 285, 286*t*
Western blotting, 260, 263*f*
Whey protein isolate, 7*f*
Whole-soybean tofu, 277
  composition, 288*t*
  fine milling, 287*f*
  grinding-treatment, 282
  making, 280
  okara, 284*t*
  sensory evaluation, 288*t*
  transglutaminase, 284*t*, 285*t*
  water-to-bean ratios, 285, 286*t*

In Chemistry, Texture, and Flavor of Soy; Cadwallader, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

WRA. *See* Water retention ability
Wu and 7S and 11S soy proteins separation, 73, 79*t*

Y

Yellow proto variety of soybeans, 116*t*
Yumeminori, 261*f*, 262*f*, 263*f*

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): December 14, 2010 | doi: 10.1021/bk-2010-1059.ix002